FUNCTIONAL ANALYSIS OF ACETYLTRANSFERASES IN PLANT POLYAMINE METABOLISM

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

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May 2017

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Cornell University 2017

Polyamines, small aliphatic amines that are ubiquitously found in almost all cellular organisms, are involved in a wide variety of physiological processes. Putrescine, which is produced from arginine, serves as a metabolic precursor for longer polyamines, including spermidine and spermine. In plants, biosynthesis of polyamines is induced in response to pathogen infection, as well as other abiotic stresses. Polyamine acetylation, which has important regulatory functions in mammalian cells, has also been observed in several plant species. Here I show that *Arabidopsis thaliana* (Arabidopsis) N-ACETYLTRANSFERASE ACTIVITY1 (NATA1) not only catalyzes acetylation of ornithine to N^{δ}-acetylornithine, but also putrescine to *N*-acetylputrescine. NATA2, encoded by a homolog of *NATA1* that is directly adjacent in the Arabidopsis genome, efficiently acetylates both ornithine and spermine.

The NATA1 and NATA2 acetyltransferases compete for polyamine metabolic flux and, hence, reduce H_2O_2 formation via polyamine oxidation. By doing so, they play a regulatory function in plants under stress. More specifically, the induction of *NATA1* and *NATA2*, by jasmonate and heat stress, respectively, reduces H_2O_2-dependent defenses in Arabidopsis and makes plants more susceptible to biotrophic pathogens, such as *Pseudomonas syringae*. This is shown by the fact that *nata1* knockout mutants are more resistant to a *P. syringae* strain DC3000. By producing

coronatine, an effector molecule that mimics jasmonate signaling, *P. syringae* highjacks the polyamine acetylation mechanism described above. Arabidopsis *nata2* knockout mutants showed similarly increased resistance to *P. syringae* under heat stress. Moreover, the chewing herbivore *Spodoptera exigua* also performed less well on *nata2* knockout mutants under heat stress. Taken together, NATA1 and NATA2 highlight the regulatory function of polyamine acetylation, as well as the controlling role polyamines play in crosstalk between jasmonate signaling, heat stress, and salicylate signaling.

Acetylated polyamines also provide an alternative pathway for polyamine formation. Duplication and neofunctionalization of *ADC1* and *NATA1*, which co-occur in a small number of plant species in the Brassicaceae, constitute an alternative pathway in which ADC1, one of two known arginine decarboxylases in Arabidopsis, converts N^{δ}-acetylornithine to *N*-acetylputrescine. Subcellular localization data indicate that ADC1 and NATA1 share a common substrate pool, as they are both localized in the endoplasmic reticulum.

BIOGRAPHICAL SKETCH

Yann-Ru Lou received her M.Sc. and Bachelor's degree in Forestry and Resource Conservation from National Taiwan University. Although she was always excited about science and research, as indicated by the many natural science camps, science competitions and the high school research internship she participated in, it is at this stage that she developed a strong love for plant secondary metabolites, which caused her to abandon her original plan of becoming a professional magician. In 2011, Yann-Ru Lou was admitted into the Ph.D. program at Department of Plant Biology, Cornell University. After a year of rotation, she joined Dr. Georg Jander's lab at Boyce Thompson Institute and focused on characterizing plant polyamine acetyltransferases and understanding their role in plant defenses against biotic stresses.

Dedicated to my family

ACKNOWLEDGMENTS

I would like to express the deepest appreciation to my committee chair Professor Georg Jander for his continuous support, inspiration and encouragement. Without his guidance and persistent help this dissertation would not have been possible. I would also like to extend my sincere thanks to my other committee members, Professor Gregory Martin and Professor Jian Hua, for their guidance and advice.

I thank many present and former lab members in the Jander lab, especially Dr. Adio Wale, Dr. Melike Bor, Dr. Jian Yan and Ms. Aileen Preuß for their contribution on this project. I also want to express my thanks to Ms. Hannah Powell for spending her beautiful summer days in lab with me. I am grateful to my collaborators, Professor Paul Morris and his lab member Ms. Sheaza Ahmed, Professor Katayoon Dehesh and her former lab member Dr. Yanmei Xiao, and Professor Daniel Voytas and his former lab member Dr. Yiping Qi. In addition, a sincere thank you to Dr. Meena Haribal and Dr. Navid Mohaved for assistance with mass spectrometry assays.

I also want to express my thanks to the National Science Foundation for providing grants to our research, and Teaching Assistantships from the Plant Biology Program as well as the Department of Ecology and Evolutionary Biology for supporting me through graduate school. I also thank my students, whose enthusiasm for pursuing knowledge frequently reignites my excitement for plant research. I wish there were room to express my thanks, individually, to all of my friends and colleagues at BTI and Cornell, who were a great pleasure to study with, work with and/or just hang out and have fun with.

Finally, I want to give special thanks to my parents Ms. Shu-Mei Lai and Dr. Shyi-Neng Lou, for their love and endless support. I considered thanking my boyfriend, Kan-Heng Lee, for his love and support, too, but I am too shy for putting it down in black and white.

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NATA	*N*-acetyltransferase activity
SSAT	Spermidine/spermine-N^1-acetyltransferase
ADC	Arginine decarboxylase
ODC	Ornithine decarboxylase
AIH	Agmatineiminohydrolase
CPA/NLP	Carbamoylputrescine amidase
SPDS	Spermidine synthase
SPMS	Spermine synthase
SAMDC	S-adenosylmethionine decarboxylase
PAO	Polyamine oxidase
CuAO	Copper amine oxidase

CHAPTER ONE

OVERVIEW OF POLYAMINE METABOLISM IN PLANTS

Polyamines are ubiquitous metabolites in almost all living cells. In plants, the most abundant polyamines are the diamine putrescine, the triamine spermidine, and the tetraamines, spermine and thermospermine (Fig. 1.1). They not only act as sinks of assimilated nitrogen but also play a central role in many aspects of plant physiology, from controlling xylem differentiation to regulating abiotic and biotic stress responses (Cona *et al.*, 2006; Moschou *et al.*, 2012; Tavladoraki *et al.*, 2012). Polyamines can be present in free and conjugated, soluble or insoluble forms (Luo *et al.*, 2009; Kaur *et al.*, 2010; Onkokesung *et al.*, 2012)

The biosynthesis and catabolism of plant polyamines

In most plants, putrescine is synthesized from arginine in two parallel pathways: by decarboxylation of ornithine, via ornithine decarboxylase (ODC; EC 4.1.1.17), or by arginine decarboxylase (ADC; EC 4.1.1.19), with agmatine as intermediate (Fig. 1.2). The latter pathway requires two additional sequential enzymatic steps, agmatine iminohydrolase (AIH; EC 3.5.3.12) and carbamoylputrescine amidase (CPA or NLP; EC 3.5.1.53) (Alcazar *et al.*, 2010; Tiburcio *et al.*, 2014). Similar to what is observed in many microorganisms, synthesis of putrescine from arginine in plants also may be catalyzed by a combination of ADC and arginine amidohydrolase (EC 3.5.3.12) (Ariyaratne, 2014). Putrescine then serves as a precursor for synthesizing higher polyamines. Spermidine is synthesized when an aminopropyl group from decarboxylated S-adenosylmethionine is transferred to putrescine by spermidine synthase (SPDS; EC 2.5.1.16). To form spermine and thermospermine, another aminopropyl group from the same source is added to spermidine on N^8 or N^1 position by spermine synthase (SPMS; EC 2.5.1.22) and thermospermine synthase (EC 2.5.1.79), respectively (Knott *et al.*, 2007; Tiburcio *et al.*, 2014). The aminopropyl group, however, is removed from decarboxylated S-adenosylmethionine by decarboxylated S-adenosylmethionine decarboxylase (SAMDC; EC 4.1.1.50) (Bagni and Tassoni, 2001).

Through genome comparisons and mutant screens, most enzymes of the basal polyamine biosynthesis pathway have been identified in Arabidopsis. Two ADC genes, *ADC1* (At2G16500) and *ADC2* (At4G34710), are known in Arabidopsis and the encoded ADC enzymes catalyze the formation of agmatine as the first and rate-limiting step in polyamine biosynthesis (Kumar *et al.*,

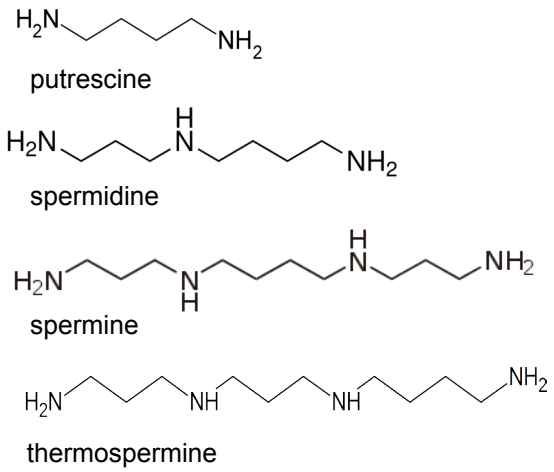

Figure 1.1. Structures of four common plant polyamines. All of them are sharing the same backbone from putrescine, the common precursor for polyamines.

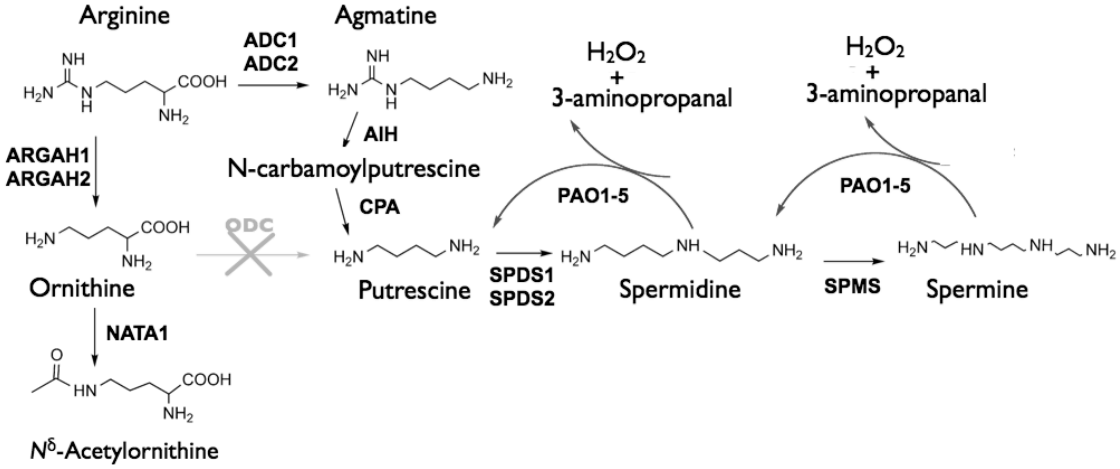

Figure 1.2. Pathways of polyamine synthesis and catabolism in Arabidopsis. Known Arabidopsis enzymes are indicated in bold. Ornithine decarboxylase (ODC) is found in most other plants, but is not present in Arabidopsis.

1997; Watson *et al.*, 1998; Hanfrey *et al.*, 2001). Hence, overexpression of *ADC* in Arabidopsis is sufficient for induced putrescine accumulation (Alcazar *et al.*, 2005). Genes encoding AIH and CPA have also been identified (Janowitz *et al.*, 2003; Piotrowski *et al.*, 2003). Three genes, *SPDS1* (AT1G23820) and *SPDS2* (AT1G70310) and *SPDS3* (At5G53120), were found to encode proteins similar to both SPDS and SPMS in other organisms. Later research clarified that only proteins encoded by *SPDS1* and *SPDS2* possess SPDS activity, whereas the *SPDS3*-encoded protein showed SPMS activity (Panicot *et al.*, 2002). Moreover, these three enzymes are believed to form a heterodimer metabolon, localized preferentially inside the nucleus (Panicot *et al.*, 2002; Belda-Palazon *et al.*, 2012). In addition, a thermospermine synthase (EC 2.5.1.79), encoded by *ACL5* (At5G19530), has also been identified after the enzyme product, thermospermine, an isomer of spermine, was detected in Arabidopsis (Hanzawa *et al.*, 2000; Knott *et al.*, 2007).

However, despite the fact that two arginases, *ARGAH1* (At4g08900) and *ARGAH2* (At4g08870) (Brownfield *et al.*, 2008), were found to catalyze the formation of ornithine from arginine, no genomic sequence homologous to known *ODC* genes has been found in Arabidopsis. An absence of enzyme activity has also been reported (Hanfrey *et al.*, 2001). Thus, Arabidopsis should make polyamines only via the agmatine branch of the pathway shown in Fig. 1.2. In fact, the arginine-dependent pathway has been suggested to be plant- and bacteria-specific. The pathway in plants has been suggested to be derived from a cyanobacterium, the progenitor of chloroplasts, via endosymbiotic gene transfer (Illingworth *et al.*, 2003; Kusano *et al.*, 2008), and its existence in invertebrates is still under debate (Li *et al.*, 1994; Reis and Regunathan, 2000; Kusano *et al.*, 2008).

Loss of ODC activity has been reported in only a few plant species, including other Brassicaceae and the moss *Physcomitrella patens* (Fuell *et al*., 2010). On the other hand, none or only remnant genes of the arginine pathway enzymes for polyamine biosynthesis have been found in the sequenced genomes of the green algae *Chlamydomonas*, *Volvox*, and *Chlorella* (Fuell *et al.*, 2010). Plants benefit from having both the arginine and ornithine polyamine biosynthesis pathways, but can survive with either one of them alone. Interestingly, both ODC-less *P. patens* and Brassicaceae plants have two or more ADC paralogs, while the ADC-less green alga *Chlamydomonas reinhardtii* has two ODC paralogs (Galloway *et al.*, 1998; Fuell *et al.*, 2010).

Cellular homeostasis of polyamines is tightly controlled not only by polyamine biosynthesis, but also by its catabolism (Tiburcio *et al.*, 1997). Polyamine catabolism is mediated by two classes of polyamine oxidases, the copper amine oxidases (CuAOs; EC 1.4.3.6) and the flavin-containing polyamine oxidases (PAOs; EC 1.5.3.11) (Fig. 1.2) (Bagni and Tassoni, 2001; Moschou *et al.*, 2008; Alcazar *et al.*, 2010; Moschou *et al.*, 2012). CuAOs, which form homodimers, catalyze the oxidation of polyamines at the primary amino group, producing H_2O_2, NH_4^+ and 4-aminobutanol if putrescine is used as the substrate (Angelini *et al.*, 2010). CuAOs are also able to oxidize spermidine and spermine, although with lower affinity than putrescine. When breaking down spermidine and spermine, 4-aza-8-amino-octan-1-al and 4,9-diaza-dodecan-1,12-dialdehyde are formed, respectively. CuAOs occur at high levels in dicots and are the most abundant soluble protein detected in the extracellular fluids of several Fabaceae (*e.g.* pea, chickpea, lentil and soybean) (Federico and Angelini, 1991; Planas-Portell *et al.*, 2013). In Arabidopsis, ten genes encode putative CuAOs (Alcazar *et al.*, 2006; Alcazar *et al.*, 2010). Among them, only four have been characterized at the protein level (Planas-Portell *et al.*, 2013; Tiburcio *et al.*, 2014). AtCuAO1 and ATAO1 are predicted to be extracellular proteins, while AtCuAO2 and AtCuAO3 are more likely to be located in peroxisomes (Planas-Portell *et al.*, 2013). Interestingly, AtCuAO1 was strongly induced by salicylic acid (SA), while the expression of AtCuAO2 responses to methyl jasmonate (MeJA) treatment (Planas-Portell *et al.*, 2013).

PAOs, on the other hand, catalyze the oxidative deamination at the secondary amino group of polyamines (Moschou *et al.*, 2008). There are two types of PAOs: in monocots, PAOs catalyze the terminal catabolism of polyamines by oxidizing the carbon at the *endo*-side of the N^4-nitrogen of spermidine and spermine; 4-aminobutanal and N-(3-aminopropyl)-4-aminobutanal are produced respectively, in addition to 1,3-diaminopropane and H_2O_2 (Fig 1.3). In Arabidopsis, however, PAOs catalyze successive back-conversion of spermine and spermidine by oxidizing the carbon at the *exo*-side of the N^4-nitrogen, producing spermidine and putrescine, respectively, in addition to 3-aminopropanal and H_2O_2 (Fig 1.3). (Moschou *et al.*, 2012; Tavladoraki *et al.*, 2012). This PAO pathway highly resembles those found in animals. Five PAO homologs have been found in Arabidopsis. While AtPAO1 and AtPAO5 have a cytosolic localization, AtPAO2, AtPAO3 and AtPAO4, which have similar gene structure and high sequence homology to one another, are reported to be in the peroxisomes (Fincato *et al.*, 2011; Fincato *et al.*, 2012; Ahou *et al.*, 2014; Kim *et al.*, 2014).

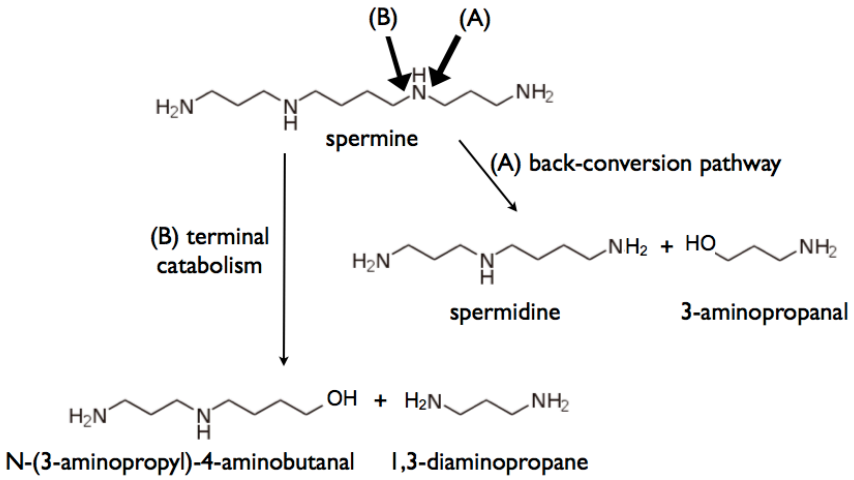

Figure 1.3. Schematic representation of spermine oxidation through (A) polyamine back-conversion pathway in Arabidopsis and (B) terminal catabolic pathway of polyamines in *Zea mays*.

The Arabidopsis PAO proteins also show differences in their activities toward specific polyamines (Tavladoraki *et al.*, 2006; Fincato *et al.*, 2011; Ahou *et al.*, 2014; Kim *et al.*, 2014). For example, AtPAO1 preferentially oxidizes spermine and its isomers, including thermospermine and norspermine, but not spermidine. Also, the activity of AtPAO1 toward N^1-acetylspermine is significantly lower than toward spermine (Tavladoraki *et al.*, 2006; Fincato *et al.*, 2011). AtPAO2, AtPAO3 and AtPAO4 oxidize both spermidine and spermine. However, while AtPAO2 shows equal activity toward spermidine and spermine, AtPAO3 is twice as active with the former as with the later. AtPAO4, on the other hand, catabolizes spermine approximately 40 times better than spermidine. Interestingly, among these three peroxisome-located PAOs, AtPAO2 showed the highest activity with N^1-acetylspermine, though this activity is still 5-fold lower than with spermine (Fincato *et al.*, 2011). AtPAO5, however, prefers N^1-acetylspermine and thermospermine as substrates over spermine and spermidine (Ahou *et al.*, 2014; Kim *et al.*, 2014). In another example of complex regulation, N^1-acetylspermine and thermospermine are suggested to function as negative effectors on AtPAO5 (Kim *et al.*, 2014).

Polyamines conjugation

Polyamines can exist both as free, soluble forms and as conjugated compounds (Alcazar *et al.*, 2010; Moschou *et al.*, 2012). One of the most common forms of conjugation are phenylpropanoid-polyamine conjugates, which are the predominant secondary metabolites in reproductive tissue. These conjugates are found in flowers, as well as on the surface of pollen grains, in Arabidopsis (Bagni and Tassoni, 2001; Fellenberg *et al.*, 2009; Grienenberger *et al.*, 2009; Kaur *et al.*, 2010). Polyamines linked to hydroxycinnamic acid dimers and/or trimers, forming hydroxycinnamic acid amides (HCAAs), are also commonly found in plant tissue (Grienenberger *et al.*, 2009; Luo *et al.*, 2009). The physiological role of HCAAs is largely unknown. However, a function in plant growth and development, including cell division, flowering, cell wall cross-linking and stress responses has been suggested (Bouchereau *et al.*, 1999; Luo *et al.*, 2009; Bassard *et al.*, 2010). In seeds, the high titer of polyamine conjugates is believed to serve as a nitrogen sink for use during germination (Moschou *et al.*, 2012). While polyamine conjugates are broadly accepted as accumulative products, they can also influence metabolism by acting as pathway intermediates, allowing turnover and translocation of polyamines to happen (Bassard *et al.*, 2010; Moschou *et al.*, 2012).

In plants, numerous polyamine acyltransferases have been identified. For instance, a member of the BAHD gene family of Arabidopsis, which encodes a spermidine hydroxycinnamoyl transferase, participating in tricoumaroyl-, tricaffeoyl-, and triferuloyl-spermidine synthesis has been identified (Grienenberger *et al.*, 2009). The same gene was also shown to be involved in the conjugation of phenylpropanoids to polyamines (Fellenberg *et al.*, 2009). Another two genes in Arabidopsis, encoding spermidine disinapoyl transferase and spermidine dicoumaroyl transferase, both belonging to the BAHD enzyme family, have also been identified by metabolic profiling of knockout mutants (Luo *et al.*, 2009). Moreover, acyltransferases involved in the biosynthesis of caffeoylputrescine and dicaffeoylspermidine have been identified in *Nicotiana attenuata* (Onkokesung *et al.*, 2012).

Developmental effects of altered polyamine metabolism

Polyamines are crucial for normal plant growth and development (Kaur-Sawhney *et al.*, 2003; Cona *et al.*, 2006; Wimalasekera *et al.*, 2011). For instance, chemically or genetically induced depletion of putrescine and/or spermidine levels is lethal in organisms such as yeast and plants (Hamasaki-Katagiri *et al.*, 1998; Imai *et al.*, 2004). In fact, with the help of chemical enzyme inhibitors and exogenous polyamine application, researchers were able to show strong correlation of polyamine levels with many aspects of plant development, including the regulation of cell proliferation, somatic embryogenesis, differentiation and morphogenesis, dormancy breaking of tubers and in seed germination, development of flowers and fruits and senescence. Also, a strong correlation between spermidine or spermine content and primary root growth is generally found in plants (Kaur-Sawhney *et al.*, 2003; Couee *et al.*, 2004; Kusano *et al.*, 2008; Takahashi *et al.*, 2010).

Knockout mutants and overexpression lines are commonly used to reveal the effects of altered polyamine metabolism. For example, transgenic Arabidopsis plants with overexpression of *ADC2* accumulated very high levels of putrescine and demonstrated dwarfism and delayed flowering (Alcazar *et al.*, 2005). This effect was likely due to reduced GA levels in the *ADC2* overexpressing lines, since dwarfism could be rescued by exogenously applying GA (Alcazar *et al.*, 2005). Consistently, Arabidopsis mutants with spermidine and spermine defects showed early proliferative arrest of apical inflorescence meristems (Hanzawa *et al.*, 2000). In fact, double knockouts of SPDS (*spds1 spds2* mutants) are embryo lethal, with the embryo being

arrested in the heart-shape stage (Imai *et al.*, 2004). Double knockouts of ADC (*adc1 adc2* mutants) are also embryo lethal, indicating that having at least one functional *ADC* gene is essential for seed development (Urano *et al.*, 2005). A mutant Arabidopsis lacking thermospermine synthase (ACL5), has a phenotype with severely shortened stature compared to the wild type. Reduced length of inflorescent stems, thickening of veins in rosette leaves and excessive proliferation of xylem vessels in *acl5* mutants suggest that thermospermine is needed to suppress xylem formation (Knott *et al.*, 2007). Interestingly, the *pao5* mutant showed similar growth defects as *acl5* mutants, suggesting that the catabolism of thermospermine is of equal importance (Kim *et al.*, 2014).

In fact, evidence suggests that the metabolic flux going through the polyamine pathway is of equal, if not greater, importance when it comes to development and related differentiation. With the use of enzyme inhibitors, apoplastic CuAOs and PAOs have been shown to play a key role in influencing cell wall architecture and maturation, as well as programmed cell death (Cona *et al.*, 2006; Angelini *et al.*, 2010; Tisi *et al.*, 2011; Tavladoraki *et al.*, 2016). The generation of H_2O_2 after these enzyme reactions is frequently regarded as the molecular mechanism behind the regulatory function. Moreover, this regulation is not limited to development, but can also be seen in plants responding to abiotic and biotic stresses (Yoda *et al.*, 2006; Moschou *et al.*, 2008; Yoda *et al.*, 2009; Angelini *et al.*, 2010; Tavladoraki *et al.*, 2016).

Polyamine involvement in plant abiotic stress responses

Polyamines are also known to enhance plant tolerance to environmental stresses such as salinity, chilling, drought and potassium deficiency (Urano *et al.*, 2003; Alcazar *et al.*, 2010; Wimalasekera *et al.*, 2011). Increased polyamine accumulation is observed in plants subjected to abiotic stress, including drought, salinity, heat, and cold (Alcazar *et al.*, 2006). ADC is thought to be the key regulatory step in this response. In Arabidopsis, *ADC1* is induced mainly by cold treatment, while *ADC2* is induced by drought and salt stress (Hummel *et al.*, 2004; Alcazar *et al.*, 2010). Arabidopsis *ADC* mutants (*adc1* and *adc2*), on the other hand, showed reduced putrescine accumulation under cold stress and lower freezing tolerance, which can be rescued by exogenous supplement of putrescine (Cuevas *et al.*, 2008). Interestingly, the content of spermidine and spermine remain constant or even decrease in response to low temperature, despite an enhanced expression of *SAMDC2* (Cuevas *et al.*, 2008).

Expression of *SPDS* and *SPMS* genes, however, also is induced by several types of abiotic stress (Alcazar *et al.*, 2006). In addition, overproduction of *SPDS* from *Cucurbita ficifolia* increases Arabidopsis tolerance of freezing and other stresses (Kasukabe *et al.*, 2004). On the hand, the *spms acl5* double mutant exhibited less tolerance to drought and salinity, implying that, although spermine is not required for cell survival, it has a role in stress resistance (Yamaguchi *et al.*, 2006; Yamaguchi *et al.*, 2007).

When it comes to heat stress, increased polyamine accumulation also has been frequently observed (Roy and Ghosh, 1996; Alcazar *et al.*, 2006; Cheng *et al.*, 2009; Sagor *et al.*, 2013; Shen *et al.*, 2016). In fact, with the induction of *SPMS*, *SAMDC2*, and *ADC2*, the levels of spermine, putrescine, and spermidine correspondingly increased upon heat stress in Arabidopsis (Sagor *et al.*, 2013). A positive correlation between the level of endogenous spermine concentration and upregulation of heat shock proteins (HSPs), as well as heat shock transcription factors, eventually leading to better heat tolerance, has also been reported (Sagor *et al.*, 2013). This could be due to the stabilizing role of polyamines. Spermine, in particular, acts on the mRNA of heat stress responsive genes under high temperature (Shen *et al.*, 2016).

Role of polyamines in biotic stress responses

Up-regulation of polyamine synthesis and/or catabolism has been reported in plants infected with a variety of viral, bacterial, and fungal pathogens (Kusano *et al.*, 2008; Hussain *et al.*, 2011). As stated before, polyamine catabolism not only regulates polyamine levels in plants, but also contributes to signaling networks via H_2O_2 production and alters many aspects of plant physiology (Marina *et al.*, 2008; Angelini *et al.*, 2010).

In animal cells, it is well known that H_2O_2, which is produced through oxidation of polyamines, plays a critical role in apoptosis (Tavladoraki *et al.*, 2012). A similar scenario is suggested to happen in tobacco mosaic virus (TMV)-infected *Nicotiana tabacum* plants. Yoda *et al*. (2003) reported that genes involved in polyamine biosynthesis were up-regulated as an immediate hypersensitive response (HR) to TMV infection, resulting in accumulation of spermidine and spermine in apoplastic space of infected leaves. Successive suppression of polyamine accumulation by chemical ODC and SAMDC inhibitors reduced the rate of HR. In contrast, direct infiltration of spermidine and spermine into healthy leaves induced the generation of H_2O_2 and simultaneously caused HR-like cell death. A three-fold increase of PAO activity in

the apoplast was observed during HR, while guazatine treatment, which competitively inhibits PAOs, resulted in reduced HR. In their further work, Yoda *et al*. (2006) showed that, in cultured cells elicited with TMV infection or cryptogein, an oomycete-originated elicitor, programmed cell death was partially mediated by H_2O_2 generated through polyamine catabolism in the extracellular space (Yoda *et al.*, 2006).

Furthermore, various necrotrophic and biotrophic pathogens also induced H_2O_2 production via polyamine catabolism in the extracellular space, resulting in defense responses (Cowley and Walters, 2002; Marina *et al.*, 2008; Moschou *et al.*, 2009; Angelini *et al.*, 2010; Hatmi *et al.*, 2014). In fact, after *Pseudomonas syringae* pv *tabaci* inoculation, *N. tabacum* cv *Xanthi* plants showed more than 18-fold increase in apoplastic spermine accumulation. Transiently overexpressing *Zea mays* PAO results in higher H_2O_2 titer in plant tissue and less growth of biotrophic pathogens. Also, a decrease in necrotic symptoms and increases in pectin, lignin and callose levels are observed (Moschou *et al.*, 2009).

In fact, Takahashi *et al*. (2003) pointed out a correlation between spermine oxidation in the apoplast and induction of SA-induced protein kinase (SIPK) and wound-induced protein kinase (WIPK), both being HR-associated defense-related genes. However, whether this activity is directly activated by spermine or hydrogen-peroxide mediated is still under debate (Takahashi *et al.*, 2003; Yoda *et al.*, 2006.)

Similar functions of polyamines have also been observed in Arabidopsis. Exogenous supply of spermine induces a similar subset of genes to HR (Mitsuya *et al.*, 2009), and the catabolism of thermospermine has been shown to contribute to Arabidopsis resistance against *P. viridiflava* (Marina *et al.*, 2013). In fact, in a transient overexpression line of *SAMDC1*, while accumulating enhanced spermine levels, Arabidopsis gained greater resistance against both the oomycete *Hyaloperonospora arabidopsidis* and the bacterium *P. syringae* (Marco *et al.*, 2014). A similar transient overexpression of *SPMS* also lead to increased spermine level, higher expression of defense genes, and increased resistance toward *P. viridiflava*. The enhanced resistance can be partly counteracted with a chemical PAO inhibitor, indicating that polyamine catabolism is involved (Gonzalez *et al.*, 2011).

Defense-induced production of conjugated polyamines has been reported in several plants (Biondi *et al.*, 2001). However, the function of these compounds in plant defense remains to be

elucidated. Conjugated polyamines are particularly abundant in reproductive tissue, which tends to be well-defended in plants. Both generalist (*Spodoptera littoralis*) and specialist (*Manduca sexta*) caterpillars show improved growth when feeding from *N. attenuata* in which expression of a regulatory MYB transcription factor that regulates formation of conjugated polyamines was silenced (Kaur *et al.*, 2010). Thus, it was hypothesized that phenylpropanoid-polyamine conjugates have a direct role in defense against lepidopteran herbivory.

Jasmonate-mediated regulation of polyamine metabolism

JA, a derivative of linolenic acid synthesized via the octadecanoid pathway, and its methyl ester (methyl JA, MeJA) are ubiquitous cyclopentanone compounds. The JA-regulated signaling pathway mediates many responses in plants, including ones elicited mechanical wounding, necrotrophic pathogens, and herbivory (Kunkel and Brooks, 2002). Insect feeding and mechanical wounding lead to the formation of JA and bioactive conjugates. Among these, JA-isoleucine (JA-Ile) conjugates, formed during defense elicitation, bind to the E3-ubiquiting ligase SCF^{COI1}, leading to the targeted degradation of JAZ repressor proteins and transcription of early JA-responsive genes (Chini *et al.*, 2007; Thines *et al.*, 2007).

There are examples of jasmonate-mediated induction of free and conjugated polyamines in a large number of plant species (Biondi *et al.*, 2001; Walters *et al.*, 2002). Together these results indicate that polyamines play a common, perhaps ubiquitous role in the JA-induced defense response of plants. In Arabidopsis, both mechanical wounding and treatment with MeJA increase the expression of *ADC2*, but not other polyamine biosynthesis genes. Consistent with the enhanced expression of *ADC2*, wounding causes a transient increase in putrescine abundance in Arabidopsis, both locally and systemically (Perez-Amador *et al.*, 2002).

Regulatory function of polyamine acetylation

Mammalian spermine/spermidine acetyltransferase (SSAT) can catalyze a variety of polyamine acetylation reactions, forming *N*-acetylspermine and *N*-acetylspermidine, as well as catalyzing the formation of N^1N^{12}-diacetylspermine from N^1-acetylspermine (Ignatenko *et al.*, 1996; Tavladoraki *et al.*, 2012). SSAT is highly regulated at the level of transcription, translation, and protein stability (Casero and Pegg, 1993). Acetylated polyamines, with reduced positive charge, are able to pass through membranes, resulting in regulatory effects on many aspects of animal metabolism (Tavladoraki *et al.*, 2012).

SSAT enzymes with varied substrates and functions also have been extensively studied in bacteria and yeast (Liu *et al.*, 2005; Bai *et al.*, 2011; Filippova *et al.*, 2015), but not yet in plants. Acetylated polyamines, however, in particular N^1-acetylspermine, N^1-acetylspermidine, N^8-acetylspermidine, have been occasionally found in plants (Table 1.1) (Hennion *et al.*, 2012). In Arabidopsis, N^1-acetylspermidine is as abundant as spermidine in both roots and above-ground tissue (Kamada-Nobusada *et al.*, 2008). However, the metabolic function of acetylated polyamines in Arabidopsis and other plants has not been investigated.

N-acetylputrescine has also been reported in *Daucus carota*, *Nicotiana plumbaginifolia*, and *Datura stramonium* cell cultures (Table 1.1) (Mesnard *et al.*, 2000; Fliniaux *et al.*, 2004). Nevertheless, pathways involved in *N*-acetylputrescine generation and degradation have not been identified, nor has there been further research to identify the function of *N*-acetylputrescine in plant metabolism.

Table 1.1. Acetylated polyamines have been sporadically found in plants.

Species	**Family**	**Acetylpolyamine**	**Reference**
Arabidopsis thaliana	Brassicaceae	N^5-acetylornithine N^1-acetylspermidine	Adio *et al.*, 2011 Kamada-Nobusada *et al.*, 2008
Arabidopsis shokei	Brassicaceae	N^5-acetylornithine	Adio *et al.*, 2011
Arabidopsis suecica	Brassicaceae	N^5-acetylornithine	Adio *et al.*, 2011
Capsella rubella	Brassicaceae	N^5-acetylornithine	Adio *et al.*, 2011
Pringlea antiscorbutica	Brassicaceae	*N*-acetylputrescine N^1-acetylspermine N^8-acetylspermidine	Hennion *et al.*, 2012
Ateleia glazioviana	Leguminosae	N^5-acetylornithine	Marona *et al.*, 1994
Ateleia herbert-smithii	Leguminosae	N^5-acetylornithine	Kite and Ireland, 2002
Bocoa spp.	Leguminosae	N^5-acetylornithine	Kite and Ireland, 2002

Onobrychis viciifolia	Leguminosae	N^{δ}-acetylornithine	Brown and Fowden, 1966
Phaseolus vulgaris		N^{δ}-acetylornithine	Zacharius, 1970
Corydalis ochotensis	Papaveraceae	N^{δ}-acetylornithine	Manske, 1937
Brachypodium sylvaticum	Poaceae	N^{δ}-acetylornithine	Fowden, 1958
Poa kerguelensis	Poaceae	N^{1}-acetylspermine N^{8}-acetylspermidine	Hennion *et al.*, 2012
Poa annua	Poaceae	*N*-acetylputrescine N^{1}-acetylspermine N^{8}-acetylspermidine	Hennion *et al.*, 2012
Poa cookii	Poaceae	*N*-acetylputrescine N^{1}-acetylspermine N^{8}-acetylspermidine	Hennion *et al.*, 2012
Ranunculus biternatus	Ranunculaceae	N^{1}-acetylspermine N^{8}-acetylspermidine	Hennion *et al.*, 2012
Ranunculus pseudotrullifolius	Ranunculaceae	N^{1}-acetylspermine N^{8}-acetylspermidine	Hennion *et al.*, 2012
Ranunculus moseleyi	Ranunculaceae	*N*-acetylputrescine N^{1}-acetylspermine N^{8}-acetylspermidine	Hennion *et al.*, 2012
Bistorta bistortoides	Polygonaceae	N^{δ}-acetylornithine	Lipson *et al.*, 1996
Datura stramonium	Solanaceae	*N*-acetylputrescine	Fliniaux *et al.*, 2004
Nicotiana plumbaginifolia	Solanaceae	*N*-acetylputrescine	Mesnard *et al.*, 2000
Acaena magellanica	Rosaceae	N^{1}-acetylspermine N^{8}-acetylspermidine	Hennion *et al.*, 2012

Taraxacum erythrospermum	Asteraceae	N^1-acetylspermine N^8-acetylspermidine	Hennion *et al.*, 2012

Reference

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CHAPTER TWO

ARABIDOPSIS NATA1 ACETYLATES PUTRESCINE AND DECREASES DEFENSE-RELATED HYDROGEN PEROXIDE ACCUMULATION[1, 2]

Abstract

Biosynthesis of the polyamines putrescine, spermidine and spermine is induced in response to pathogen infection of plants. Putrescine, which is produced from arginine, serves as a metabolic precursor for longer polyamines, including spermidine and spermine. Polyamine acetylation, which has important regulatory functions in mammalian cells, has been observed in several plant species. Here we show that *Arabidopsis thaliana* (Arabidopsis) N-ACETYLTRANSFERASE ACTIVITY1 (NATA1) catalyzes acetylation of putrescine to *N*-acetylputrescine and thereby competes with spermidine synthase for a common substrate. *NATA1* expression is strongly induced by the plant defense signaling molecule jasmonic acid and coronatine, an effector molecule produced by DC3000, a *Pseudomonas syringae* strain that initiates a virulent infection in Arabidopsis ecotype Columbia-0. DC3000 growth is reduced in *nata1* mutant Arabidopsis, suggesting a role for NATA1-mediated putrescine acetylation in suppressing antimicrobial defenses. During infection by *P. syringae* and other plant pathogens, polyamine oxidases use spermidine and spermine as substrates for the production of defense-related H_2O_2. Compared to wildtype Columbia-0 Arabidopsis, the response of *nata1*mutants to *P. syringae* infection includes reduced accumulation of acetylputrescine, greater abundance of non-acetylated polyamines, elevated H_2O_2 production by polyamine oxidases, and higher expression of genes related to pathogen defense. Together, these results are consistent with a model whereby *P. syringae* growth is improved in a targeted manner through coronatine-induced putrescine acetylation by NATA1.

[1]All work in this chapter has been published in *Plant Physiology* **171:** 1443-1455, DOI:10.1104/pp.16.00446

[2]Yann-Ru Lou designed and performed most of the enzyme characterization experiments, determined the plant defense responses after pathogen infection, and contributed to replicates for pathogen growth experiments. Article was written with help from Dr. Melike Bor and Dr. Georg Jander.

Introduction

Plant polyamines, including putrescine, spermidine, and spermine (Fig. 2.1A), accumulate in response to a variety of biotic and abiotic stresses (Walters, 2003; Minocha *et al*., 2014). Synthesis of putrescine from arginine occurs via two pathways, with either ornithine or agmatine as an intermediate (Fig. 2.1B). Although there are two arginases (*ARGAH1* and *ARGAH2*) in the *Arabidopsis thaliana* (Arabidopsis) genome (Brownfield *et al*., 2008), ornithine decarboxylase (*ODC*) is apparently absent, indicating that, unlike in most other plant species, ornithine is not a direct metabolic precursor for putrescine synthesis in Arabidopsis (Hanfrey *et al*., 2001). Putrescine, in turn, is converted into spermidine through the addition of an aminopropyl group from decarboxylated *S*-adenosylmethionine by spermidine synthases (SPDS1 and SPDS2). An additional aminopropyl group is added to spermidine to form spermine by spermine synthase (SPMS).

Polyamine acetylation, which has important regulatory functions in mammalian stress responses (Pegg, 2008; Tavladoraki *et al*., 2012), also has been observed in plants. *N*-acetylputrescine was detected in *Daucus carota*, *Nicotiana plumbaginifolia*, and *Datura stramonium* (Mesnard *et al*., 2000; Fliniaux *et al*., 2004). N^1-acetylspermine, N^1-acetylspermidine, and N^8-acetylspermidine have been found in several plant species (Dufeu *et al*., 2003; Hennion *et al*., 2012). In Arabidopsis, N^1-acetylspermidine is as abundant as spermidine in both roots and above-ground tissue (Kamada-Nobusada *et al*., 2008). However, despite the apparently widespread distribution of acetylated polyamines in the plant kingdom, there has been as yet no in-depth investigation of functions of these plant metabolites and their biosynthetic enzymes.

Polyamine catabolism is mediated by two classes of polyamine oxidases, the copper amine oxidases (CuAOs; EC 1.4.3.6) and the flavin-containing polyamine oxidases (PAOs; EC 1.5.3.11) (Fig. 2.1B) (Bagni and Tassoni, 2001; Moschou *et al*., 2008; Alcazar *et al*., 2010; Moschou *et al*., 2012). CuAOs form homodimers and can catalyze the oxidation of putrescine at its primary amino group, producing H_2O_2, NH_4^+ and 4-aminobutanol (Cona *et al*., 2006; Angelini *et al*., 2010). Although they have low activity toward spermidine and spermine, CuAOs also generate H_2O_2 by breaking down these polyamines into 4-aza-8-amino-octan-1-al and 4,9-diaza-dodecan-1,12 dialdehyde, respectively (Moschou *et al*., 2012; Planas-Portell *et al*., 2013). PAOs, on the other hand, catalyze the oxidative deamination at the secondary amino group on

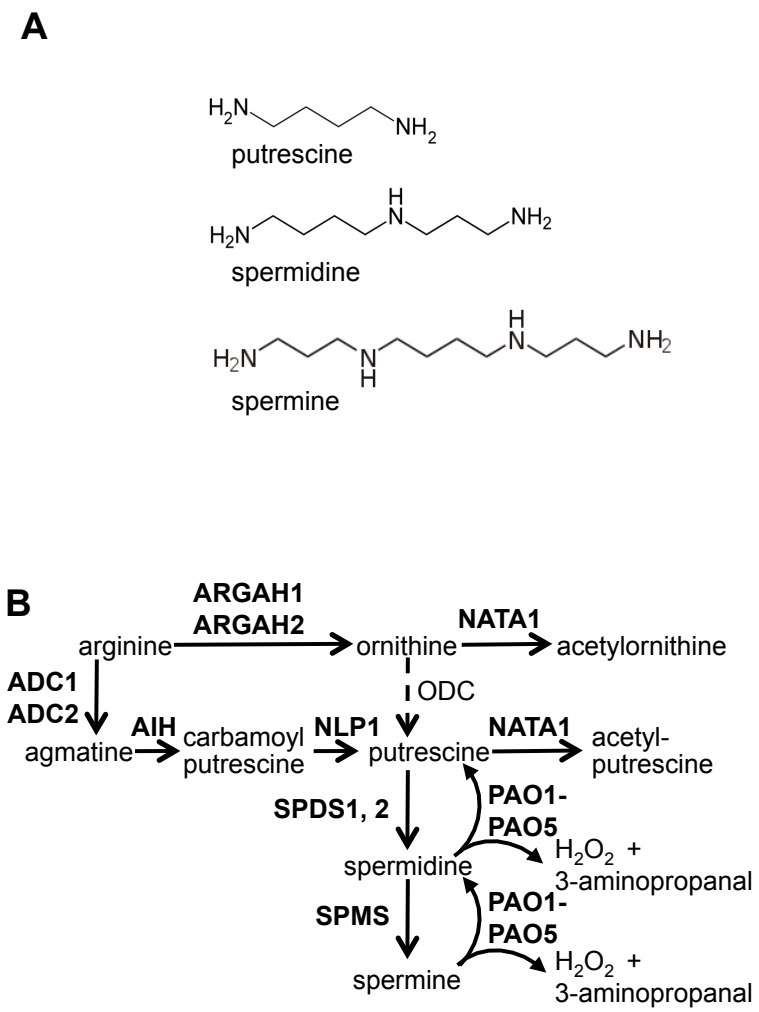


Figure 2.1. Polyamine metabolism in Arabidopsis. (A) Structures of three common plant polyamines. (B) Pathways of polyamine synthesis and catabolism in Arabidopsis. Known Arabidopsis enzymes are indicated in bold. Ornithine decarboxylase (ODC) is found in other plants, but is not present in Arabidopsis.

spermidine and spermine (Moschou *et al*., 2008). In some monocots, including maize (*Zea mays*) and barley (*Hordeum vulgare.*), PAOs catalyze the terminal catabolism of polyamines by oxidizing the carbon at the *endo*-side of the N^4-nitrogen of spermidine and spermine, producing 4-aminobutanal and *N*-(3-aminopropyl)-4-aminobutanal, respectively, in addition to 1,3-diaminopropane and H_2O_2 (Moschou *et al*., 2012; Tavladoraki *et al*., 2012). In other plant species, including Arabidopsis and rice (*Oryza sativa*), PAOs function in a polyamine back-conversion pathway; oxidizing the carbon at the *exo*-side of the N^4-nitrogen of the triamine spermidine, and the tetraamines spermine and thermospermine, thereby producing putrescine and spermidine, respectively, in addition to 3-aminopropanal and H_2O_2 (Fig. 2.1B) (Moschou *et al*., 2012; Ono *et al*., 2012; Tavladoraki *et al*., 2012; Ahou *et al*., 2014; Kim *et al*., 2014).

Five PAO homologs have been identified in Arabidopsis: Whereas PAO1 and PAO5 are cytosolic enzymes, PAO2, PAO3 and PAO4 are targeted to the peroxisomes (Kamada-Nobusada *et al*., 2008; Moschou *et al*., 2008; Ahou *et al*., 2014; Kim *et al*., 2014). Polyamine oxidation by PAOs is a major source of stress-induced H_2O_2 accumulation in plant tissue (Yoda *et al*., 2009). Both necrotrophic and biotrophic pathogens can induce H_2O_2 production via polyamine oxidation (Marina *et al*., 2008; Moschou *et al*., 2009; Angelini *et al*., 2010), and up-regulation of polyamine biosynthesis has been reported in plants infected with a variety of viral, bacterial, and fungal pathogens (Walters, 2003; Walters, 2003). Arabidopsis PAOs oxidize spermine and spermidine more efficiently than N^1-acetylspermine and N^1-acetylspermidine (Moschou *et al*., 2008; Kim *et al*., 2014), suggesting that non-acetylated polyamines are more likely to be physiologically relevant substrates of these enzymes.

Coronatine, a toxin produced by some *P. syringae* pathovars, including the commonly studied DC3000 strain (Xin and He, 2013), is a molecular mimic of the jasmonate-isoleucine conjugate that binds to the COI1 (CORONATINE INSENSITIVE 1) receptor and activates jasmonate-specific defense responses (Brooks *et al*., 2004; Fonseca *et al*., 2009). In the case of bacteria on the leaf surface, coronatine can prevent flagellin-induced stomatal closure, thereby enabling bacterial entry into the leaves (Melotto *et al*., 2006). Arabidopsis *NATA1* (*N-ACETYLTRANSFERASE ACTIVITY1*; At2g39030), which is one of the most strongly induced genes after jasmonate or coronatine treatment, is an ornithine acetyltransferase that produces N^{δ}-acetylornithine from ornithine (Adio *et al*., 2011). The *NATA2* gene (At2g39020) is directly adjacent to *NATA1* in the Arabidopsis genome and encodes a protein that is 80% identical to

NATA1. However, unlike *NATA1*, *NATA2* expression is not induced by jasmonic acid or coronatine (Adio *et al*., 2011). Instead, *NATA2* is expressed at a uniformly high level throughout Arabidopsis development (Supplemental Fig. S2.1).

Recent *in vitro* assays with NATA1 (Jammes *et al*., 2014) showed that this enzyme also acetylates 1,3-diaminopropane, which has been reported as a product of polyamine oxidation by maize PAO (Terano and Suzuki, 1978). *N*-Acetyl-1,3-diaminopropane, in turn, is an antagonist of abscisic acid-induced stomatal closure (Jammes *et al*., 2014). Thus, it is hypothesized that, by inducing diaminopropane acetylation with coronatine, *P. syringae* may inhibit stomatal closure and facilitate entry into the leaf. Once *P. syringae* are growing inside of plant tissue, coronatine promotes virulence by activating jasmonate-regulated defenses and thereby suppressing pathogen-targeted defenses (Cui *et al*., 2005; Uppalapati *et al*., 2007). Coronatine-deficient strains can grow to high concentrations in salicylic acid-deficient plants, suggesting that suppression of salicylate-dependent defense responses is a key function of this effector (Brooks *et al*., 2005). A role for the COI1 receptor in this defense suppression is demonstrated by the observation that coronatine-producing bacteria grow to a lower titer in a *coi1* mutant than in wildtype Arabidopsis (Feys *et al*., 1994; Kloek *et al*., 2001). By increasing expression of the jasmonate-responsive transcription factor *MYC2*, coronatine activates three NAC transcription factors, ANAC019, ANAC055, and ANAC072, that reduce accumulation of the signaling molecule salicylic acid, which is required for the up-regulation of many pathogen defenses in plants (Zheng *et al*., 2012). Conversely, Arabidopsis *myc2* transcription factor mutants, which are defective in jasmonate-induced defense responses, have elevated salicylic acid levels and *PR* gene expression (Laurie-Berry *et al*., 2006). Thus, coronatine-mediated up-regulation of a jasmonate-regulated Arabidopsis transcription factor promotes *P. syringae* virulence through transcriptional suppression of salicylate-mediated defenses.

Salicylic acid and H_2O_2 act synergistically to initiate both local and systemic plant responses to pathogen infection (Neuenschwander *et al*., 1995; Sharma *et al*., 1996; Alvarez *et al*., 1998; Mukherjee *et al*., 2010). A rapid increase in H_2O_2 production, initiated through the activity of NADPH oxidase, occurs as a hypersensitive response to pathogen infection in many plant species (Lamb and Dixon, 1997; Torres *et al*., 2005). In contrast, more sustained H_2O_2 accumulation during virulent pathogen infection occurs through degradation of spermidine and spermine by PAO activity and also contributes to plant defense (Marina *et al*., 2008; Moschou *et*

al., 2009; Angelini *et al*., 2010). In addition to NADPH oxidase and PAO, Class III peroxidase activity is a source of H_2O_2 that can limit bacterial growth in Arabidopsis (Mitchell *et al*., 2014).

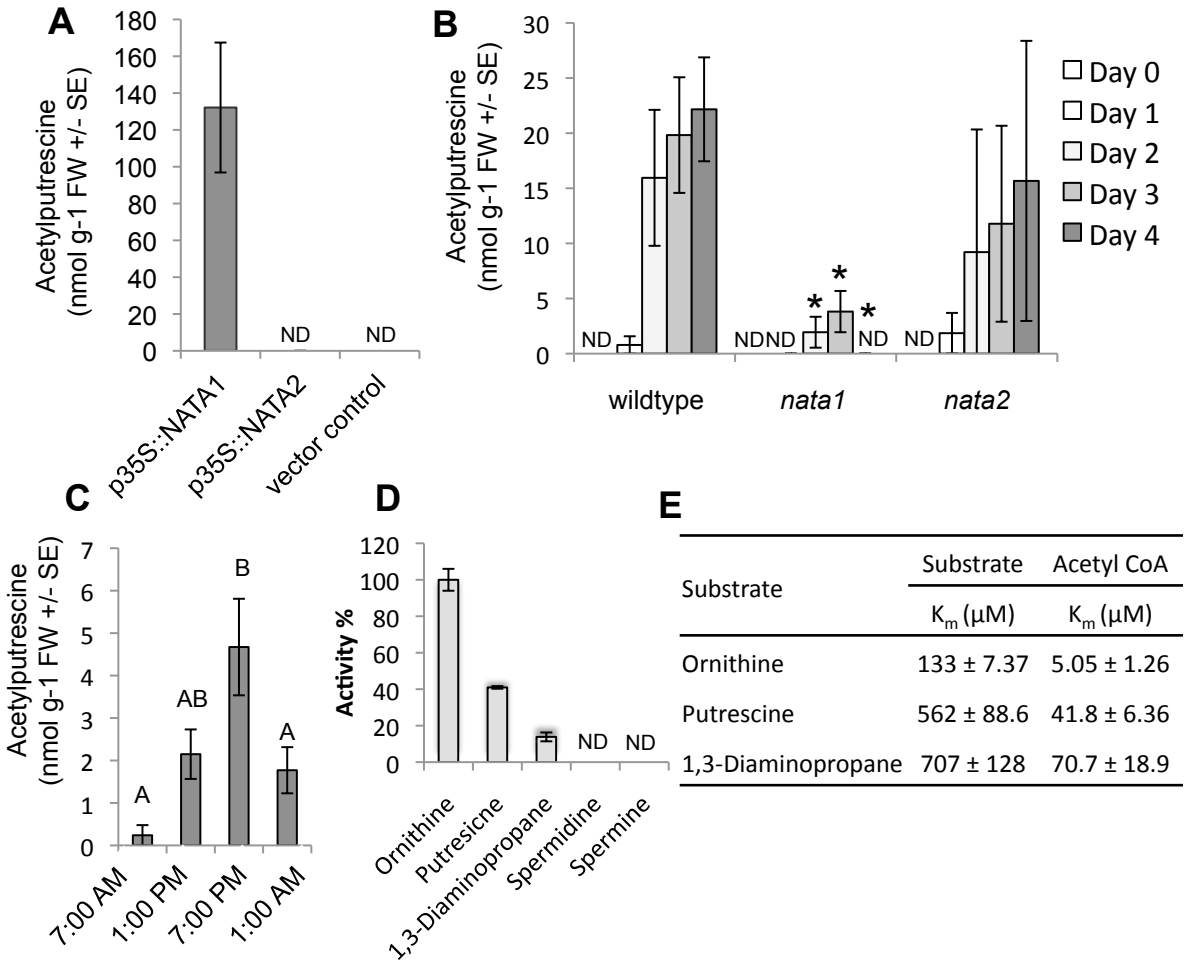
After infiltration into Arabidopsis leaves, virulent *P. syringae* DC3000 propagation was lower in *nata1* mutant than in wildtype Columbia-0 (Col-0) plants (Adio *et al*., 2011), suggesting that NATA1 might function in suppressing antibacterial defenses. We now provide an explanation for this observation by showing that, in addition to using ornithine and diaminopropane as substrates (Adio *et al*., 2011; Jammes *et al*., 2014), NATA1 functions as a putrescine acetyltransferase (Fig. 2.1B). Up-regulation of *NATA1* expression by coronatine-producing *P. syringae* DC3000 causes elevated *N*-acetylputrescine production and reduced H_2O_2 production by polyamine oxidase. Higher H_2O_2 accumulation in *nata1* mutants leads to increased expression of defense-related genes and reduced bacterial growth. Together, these observations indicate that, by inducing jasmonate-regulated polyamine acetylation, coronatine-producing *P. syringae* suppress pathogen-specific defenses.

Results

Arabidopsis NATA1 functions as a putrescine acetyltransferase

The Arabidopsis *NATA1* and *NATA2* genes are the closest homologs of known mammalian polyamine acetyltransferases (Supplemental Fig. S2.2). This similarity, together with the observation of acetylated polyamines in Arabidopsis and other plants (Mesnard *et al*., 2000; Fliniaux *et al*., 2004; Kamada-Nobusada *et al*., 2008; Hennion *et al*., 2012), suggested that NATA1 and NATA2 might be polyamine acetyltransferases. Although the predicted catalytic residues of the mammalian enzymes are conserved in the Arabidopsis NATA1 and NATA2 proteins, not all of the known spermine binding residues are present (Supplemental Fig. S2.3). Thus, Arabidopsis NATA1 and NATA2 likely acetylate amines, but the *in vivo* substrates might be different from those of the corresponding mammalian enzymes.

To determine whether NATA1 and/or NATA2 acetylate polyamines, the corresponding genes were expressed transiently in *Nicotiana benthamiana. NATA1,* but not *NATA2* expression caused accumulation of *N*-acetylputrescine (Fig. 2.2A). Similar to what was observed in the case of N^{δ}-acetylornithine (Adio *et al*., 2011), *N*-acetylputrescine accumulation was induced at 2, 3, and 4 days after methyl jasmonate treatment of wildtype Col-0 plants (Fig. 2.2B; $P < 0.05$, *t*-test relative to day 0 control). Consistent with a role for NATA1 in Arabidopsis polyamine



Substrate	Substrate	Acetyl CoA
	K_m (µM)	K_m (µM)
Ornithine	133 ± 7.37	5.05 ± 1.26
Putrescine	562 ± 88.6	41.8 ± 6.36
1,3-Diaminopropane	707 ± 128	70.7 ± 18.9

Figure 2.2. Arabidopsis NATA1 is a putrescine acetyltransferase (A) p35S::*NATA1* transient expression in *N. benthamiana* results in *N*-acetylputrescine production. Mean ± SE of N = 12-14. (B) Time course of *N*-acetylputrescine accumulation in rosette leaves after MeJA treatment. Mean ± SE of N = 3, *P < 0.05, two-tailed *t*-test relative to wildtype at the same time point. (C) Accumulation of *N*-acetylputrescine in Arabidopsis is affected by the circadian clock. Mean ± SE of n = 12-23. Plants were grown in a 16:8 light:dark cycled, with dawn at 7 AM. Different letters indicate significant differences, P < 0.05, ANOVA followed by Tukey's HSD test. (D) Relative activity of recombinant NATA1 enzyme, measured by release of CoA, in 100 mM Tris-HCl at pH 7.5 and 30°C. Substrate concentration was fixed at 5 mM and the acetyl-CoA concentration was fixed at 600 µM. Values are mean ± S.E of n = 3. (E) Kinetic constants of substrate acetylation by recombinant NATA1. For ornithine, putrescine and 1,3 diamionopropane, Km was determined by Michaelis-Menten curve with a 1 mM fixed concentration of acetyl-CoA and varying substrate concentration from 25 to 1000 µM. For acetyl-CoA, Km was determined by Michaelis-Menten curve with a 5 mM fixed concentration of substrate and varying acetyl-CoA concentration from 1.25 to 600 µM. All data are mean ± S.E of 3 independent experiments. ND = not detected.

metabolism, this induction was significantly reduced in *nata1* mutant plants (Fig. 2.2B). In contrast, a *nata2* T-DNA insertion knockout did not have a significant effect on methyl jasmonate-induced *N*-acetylputrescine accumulation. Acetylated spermine and spermidine were either not present or accumulated at concentrations below the detection limit of our assay (~20 nmol g^{-1} for acetylated spermine and spermidine added to Arabidopsis leaf extracts). Arabidopsis ecotypes with natural *nata1* knockout mutations, which were identified through the 1001 Genomes project (Bur-0, Amel-1, and Cal-1; http://1001genomes.org/; Supplemental Fig. S2.4), did not exhibit any detectable amounts of *N*-acetylputrescine and N^{δ}-acetylornithine accumulation in response to methyl jasmonate treatment. Consistent with the circadian cycle of jasmonate accumulation in plants (Goodspeed *et al*., 2012; Shin *et al*., 2012) we observed circadian regulation in the abundance of *N*-acetylputrescine (Fig. 2.2C). Maximum levels of *N*-acetylputrescine accumulation in this experiment were lower than those observed in response to methyl jasmonate or other elicitor treatments. However, given the observed circadian regulation, plant material for all subsequent induction experiments to measure gene expression or metabolite abundance was collected before 10 AM, when the background *N*-acetylputrescine abundance was low.

To confirm NATA1 catalytic activity and examine substrate preferences, *in vitro* assays were performed with Arabidopsis NATA1 protein that was purified after expression in *Escherichia coli* (Supplementary Fig. S2.5). Ornithine, putrescine, and 1,3-diaminopropane served as acetyltransferase substrates *in vitro* (Fig. 2.2D). Consistent with the absence of known spermine and spermidine binding residues in NATA1 (Supplemental Fig. S2.3), these two compounds did not serve as acetylation substrates *in vitro*. The apparent Km values for ornithine, putrescine, 1,3-diaminopropane, and acetyl-coA were measured with purified NATA1 by varying the substrate concentrations and graphing Michaelis-Menten curves (Supplemental Fig. S2.6). Consistent with the relative NATA1 acetylation activity of ornithine > putrescine > 1,3-diaminopropane (Fig. 2.2D), the relative Km values for these substrates were 1,3-diaminopropane > putrescine > ornithine (Fig. 2.2E).

Virulent DC3000 infection induces acetylputrescine accumulation in a NATA1-dependent manner

Similar to methyl jasmonate treatment (Fig. 2.2B), exogenous coronatine addition induced greater *N*-acetylputrescine accumulation in wildtype than in *nata1* mutant plants (Fig. 2.3A). Consistent with a role for coronatine in triggering putrescine acetylation, wildtype *P. syringae* strain DC3000 infiltration induced Arabidopsis *NATA1* gene expression to a higher level than DC3000 cor- (Fig. 2.3B). DC3000 infection induced *N*-acetylputrescine accumulation in *NATA1*-dependent manner (Fig. 2.4A). Prior research demonstrated that, after methyl jasmonate treatment, putrescine and spermidine are significantly more abundant in *nata1* than in wildtype Arabidopsis (Adio *et al*., 2011). Consistent with the observation that NATA1 converts putrescine to *N*-acetylputrescine, putrescine accumulated to a higher level during DC3000 infection of *nata1* mutant than wildtype plants (Fig. 2.4B). Spermidine, was slightly increased in *nata1* relative to wildtype Arabidopsis (Fig. 2.4C), and spermine showed variable effects, with significantly lower levels in *nata1* two days after *P. syringae* infiltration and significantly higher levels after four days (Fig. 2.4D). The transcript levels of spermidine and spermine synthases, *SPDS1*, *SPDS2* and *SPMS*, were induced to comparable levels in *nata1* mutant and wildtype plants after 2 days of *P. syringae* infiltration (Fig. 2.4E and 2.4F). However, transcript abundance of *SPMS* was induced to higher level in *nata1* mutant plants 4 days after pathogen challenge (Fig. 2.4G), which may indicate increased metabolic flux through this pathway.

H_2O_2 induction by *P. syringae* is increased in *nata1* mutant plants

As H_2O_2 synthesis by polyamine oxidase commonly occurs during pathogen infection (Marina *et al*., 2008; Moschou *et al*., 2009; Angelini *et al*., 2010), we determined whether this response is altered in *nata1* mutant Arabidopsis. In contrast to water-only controls, infiltration with virulent *P. syringae* strain DC3000 induced H_2O_2 to a higher level in *nata1* than in wildtype Arabidopsis (Fig. 2.5A). H_2O_2 accumulation was greatly reduced if *P. syringae* bacteria were co-infiltrated with guazatine, an inhibitor of polyamine oxidase activity (Yoda *et al*., 2003). The final concentration of guazatine in the leaves was less than 2 μM, which does not cause significant negative effects on bacterial growth *in vitro* (Supplemental Fig. S2.7). Further experiments also showed that *in planta P. syringae* growth also is not significantly inhibited by this level of guazatine infiltration. DC3000 infection induced H_2O_2 accumulation to a higher level in the *coi1* mutant than in wildtype Col-0 (Fig. 2.5B), indicating that recognition of coronatine by COI1 is required for the suppression of the H_2O_2 response. Suppression of the H_2O_2 increase by guazatine

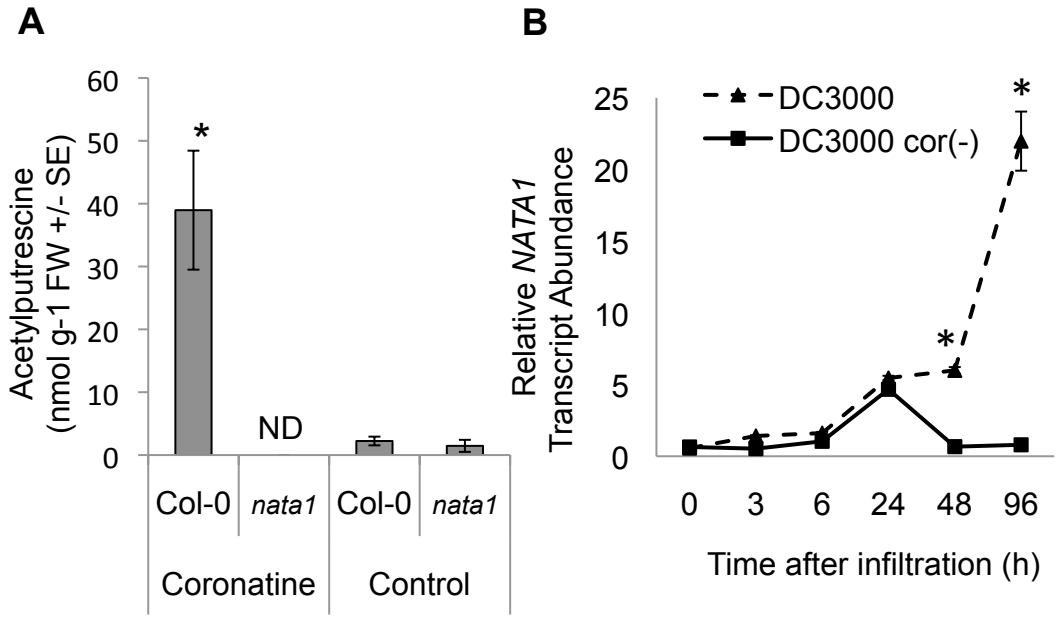


Figure 2.3. *Pseudomonas syringae* induces acetylputrescine accumulation in a NATA1-dependent manner. (A) *N*-acetylputrescine accumulation in rosette leaves 4 days after coronatine treatment. Mean ± SE of N = 12 (control) or 9 (coronatine), *P < 0.05, two-tailed *t*-test comparing Col-0 and *nata1*. ND = not detected. (B) *NATA1* gene expression in wildtype Col-0 plants infiltrated with *P. syringae* DC3000 and DC3000 cor(-). *NATA1* gene expression was measured by quantitative RT-PCR. Mean ± SE of n = 6-9, *P < 0.05, two-tailed *t*-test comparing mutant and wildtype at the same time point.

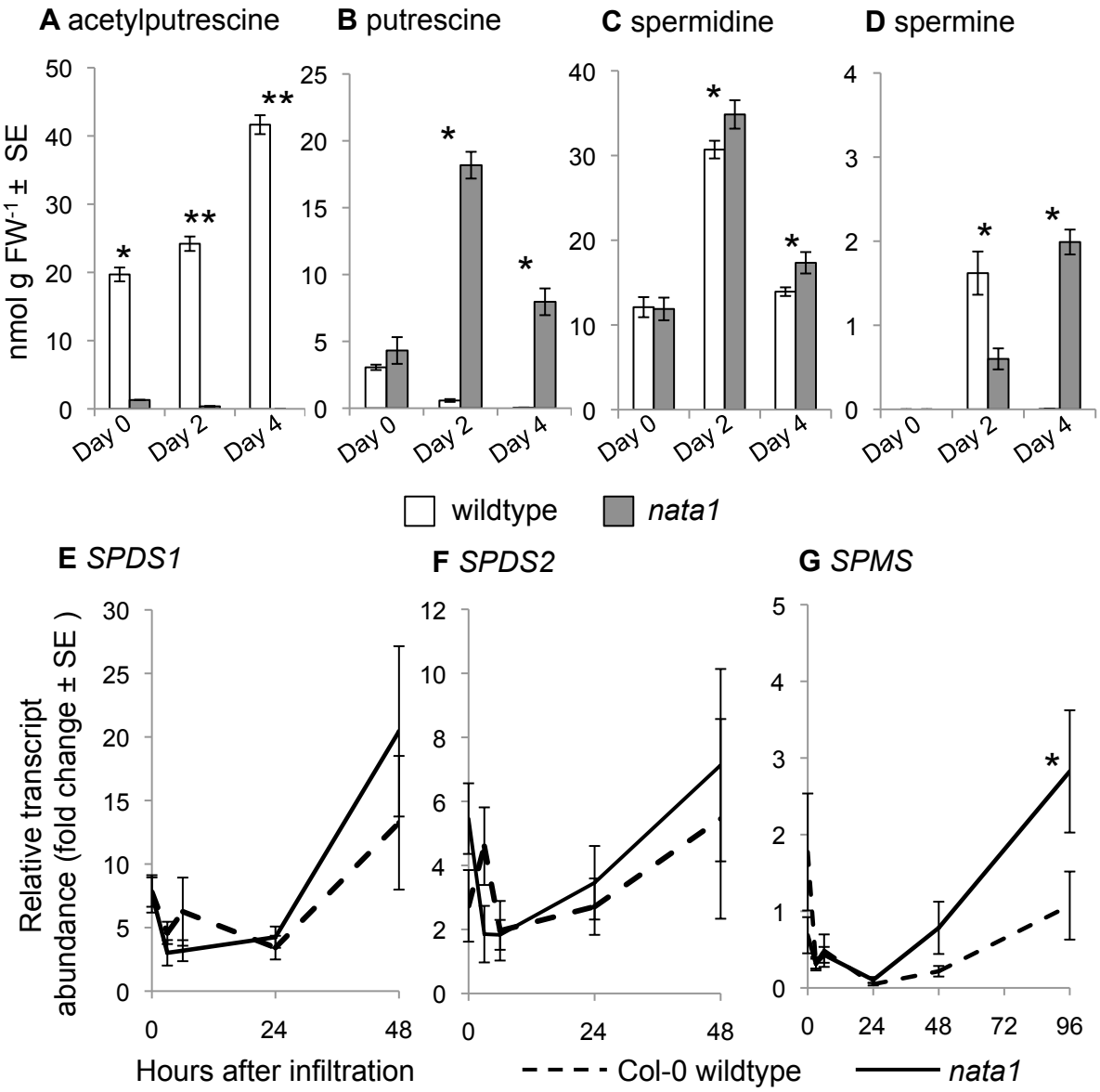


Figure 2.4. Polyamine concentrations and transcript abundance of spermidine and spermine synthases in wildtype Col-0 and *nata1* mutant Arabidopsis infiltrated with *P. syringae* DC3000. (A) *N*-acetylputrescine, (B) putrescine, (C) spermidine, and (D) spermine. FW = fresh weight. Mean ± SE of n = 6. *P < 0.05, two-tailed *t*-test. (E) *SPDS1,* (F) *SPDS2*, and (G) *SPMS* expression was measured by quantitative RT-PCR. Mean ± SE of N = 8-9, *P < 0.05, two tailed *t*-test comparing expression in wildtype and *nata1* at the same time point.

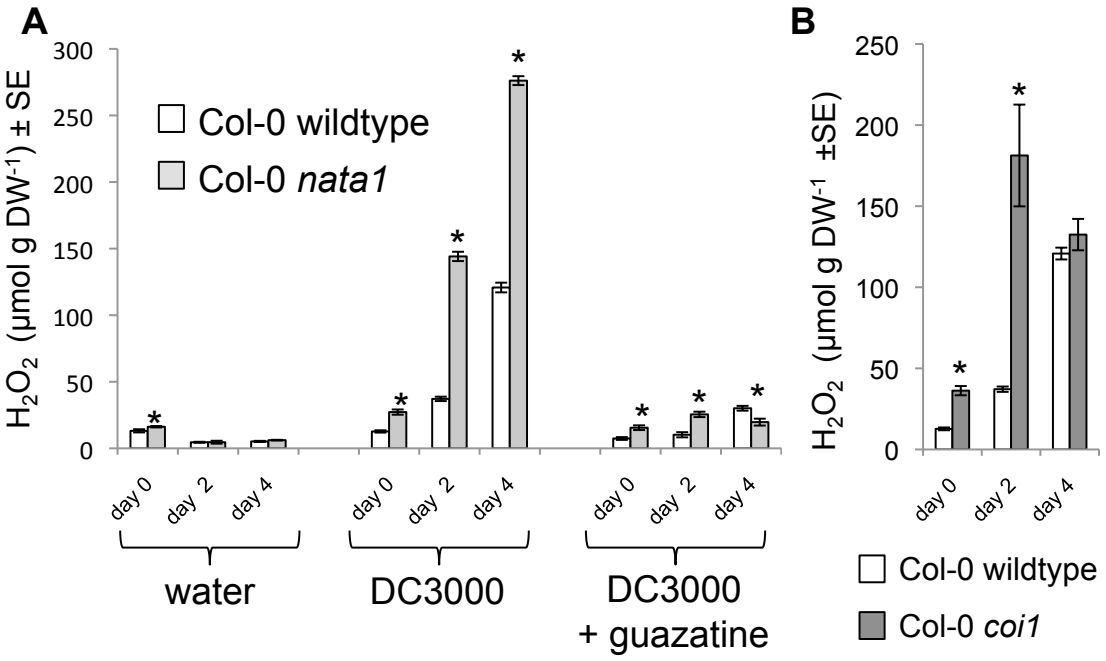


Figure 2.5. H_2O_2 induction by *P. syringae* infection. (A) H_2O_2 content of wildtype Col-0 and *nata1* mutant Arabidopsis infiltrated with water as a control, wildtype DC3000, and wildtype DC3000 together with guazatine, a polyamine oxidase inhibitor. Mean ± SE of n = 6-10. (B) H_2O_2 content in Col-0 wildtype and *coi1* mutant Arabidopsis after infiltration with DC3000. Mean ± SE of N = 6 for wildtype, N = 9 for *coi1*. *P < 0.05, two-tailed *t*-test. DW = dry weight.

suggests that most the H_2O_2 that was detected in these experiments is produced from polyamines by PAO.

PAO activity was measured in Arabidopsis extracts to determine whether the observed differences in H_2O_2 accumulation could result from different levels of enzyme activity in wildtype and *nata1* plants infiltrated with *P. syringae*. Two and four days after infection, there was a similar induction of polyamine oxidase enzymatic activity by DC3000 infiltration in the two Arabidopsis genotypes (Fig. 2.6A). Consistent with previously observed differential regulation of the five known Arabidopsis *PAO* genes (Fincato *et al*., 2012), the effects of DC3000 infection on *PAO* expression varied (Fig. 2.6). Whereas expression of *PAO1*, *PAO2*, *PAO3*, and *PAO4* was up-regulated, that of *PAO5* was not. However, no clear pattern was observed when comparing *PAO* transcript levels in the *nata1* mutant and wildtype plants. Whereas *PAO1* was expressed at a higher level in wildtype Col-0, *PAO2* and *PAO3* were expressed at a higher level in the *nata1* mutant. There were no significant differences in the case of *PAO4* and *PAO5*. Taken together, these data may indicate that differential H_2O_2 accumulation in wildtype and *nata1* plants may be caused by substrate availability rather than enzyme abundance.

Coronatine-mediated induction of *NATA1* improves *P. syringae* growth

Bacterial growth experiments were conducted to determine whether *NATA1* upregulation by coronatine benefits *P. syringae*. Four days after infiltration, bacterial counts were three-fold higher in wildtype Col-0 than in *nata1*plants (Fig. 2.7A), similar to the effect observed by Adio *et al.* (2011). In contrast, in plants treated with guazatine at the time of *P. syringae* infiltration, bacteria in *nata1* mutants did not grow to a lower final concentration than in wildtype (Fig. 2.7B), showing that H_2O_2 production by PAO, which is inhibited by guazatine, contributes to plant defense. Growth of DC3000 cor- was similar in wildtype and *nata1* Arabidopsis, indicating that at least some suppression of plant defenses by coronatine occurs in a *NATA1*-dependent manner (Fig. 2.7C).

To determine whether NATA1 activity can lead to a suppression of anti-bacterial defenses in other plants, *P. syringae* growth was assessed in *N. benthamiana* leaves that were transiently expressing *NATA1*. Unlike in the case of Arabidopsis, wildtype DC3000 effectors are recognized by *N. benthamiana* to mount a defense response (Wei *et al*., 2007; Wei *et al*., 2015).

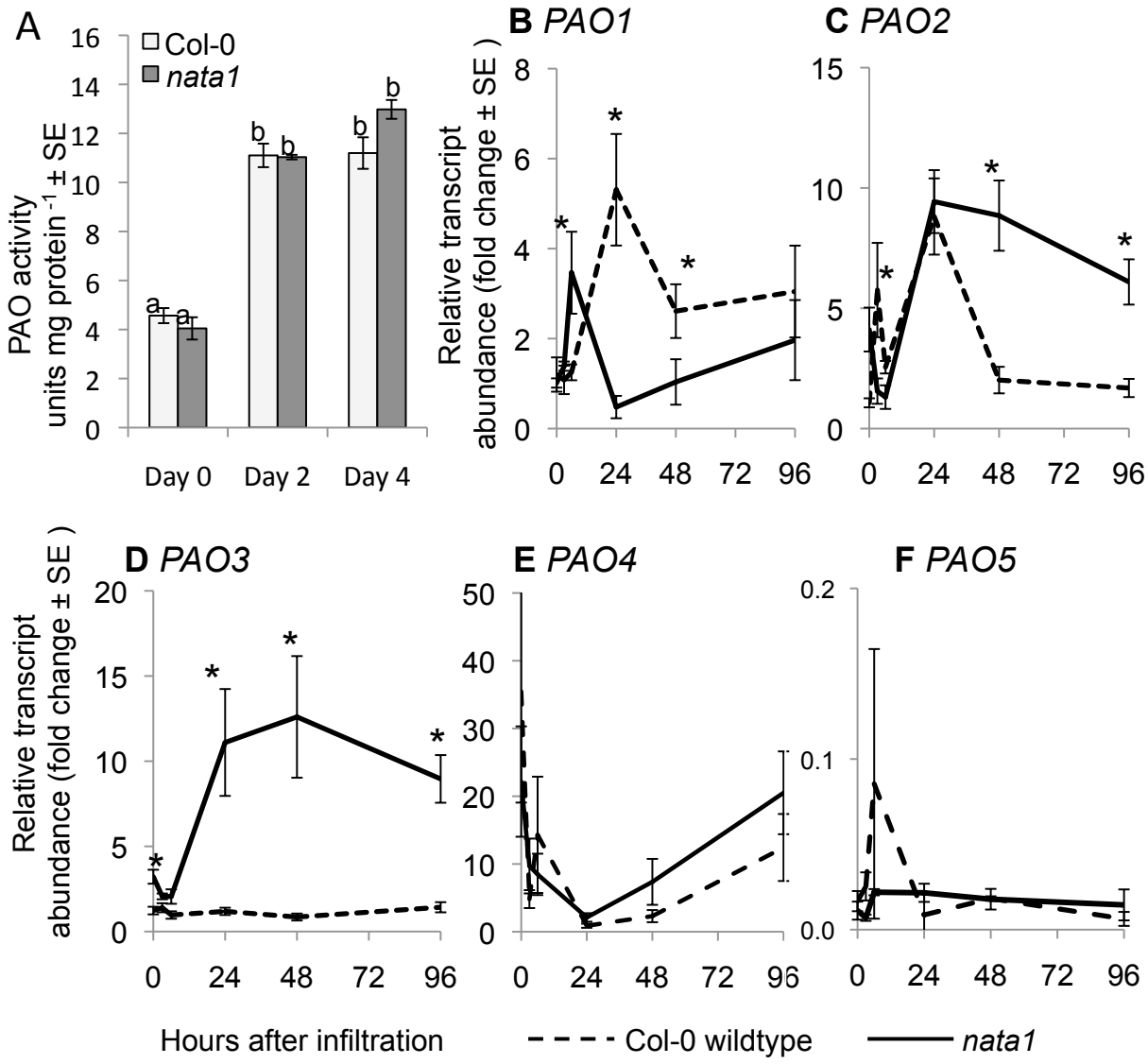


Figure 2.6. Polyamine oxidase responses to *P. syringae*. (A) Col-0 wild-type and *nata1* mutant leaves were infiltrated with *P. syringae* DC3000. Mean ± SE of n = 3. Different letters indicate significant difference, $P < 0.05$, ANOVA followed by Tukey's HSD test. Transcript abundance of polyamine oxidases (B) *PAO1,* (C) *PAO2*, (D) *PAO3,* (E) *PAO4* and (F) *PAO5* was measured by quantitative RT-PCR at the indicated times after *P. syringae* infiltration into leaves. Mean ± SE of n = 9, *$P < 0.05$, two tailed *t*-test comparing wildtype and *nata1* expression at the same time point.

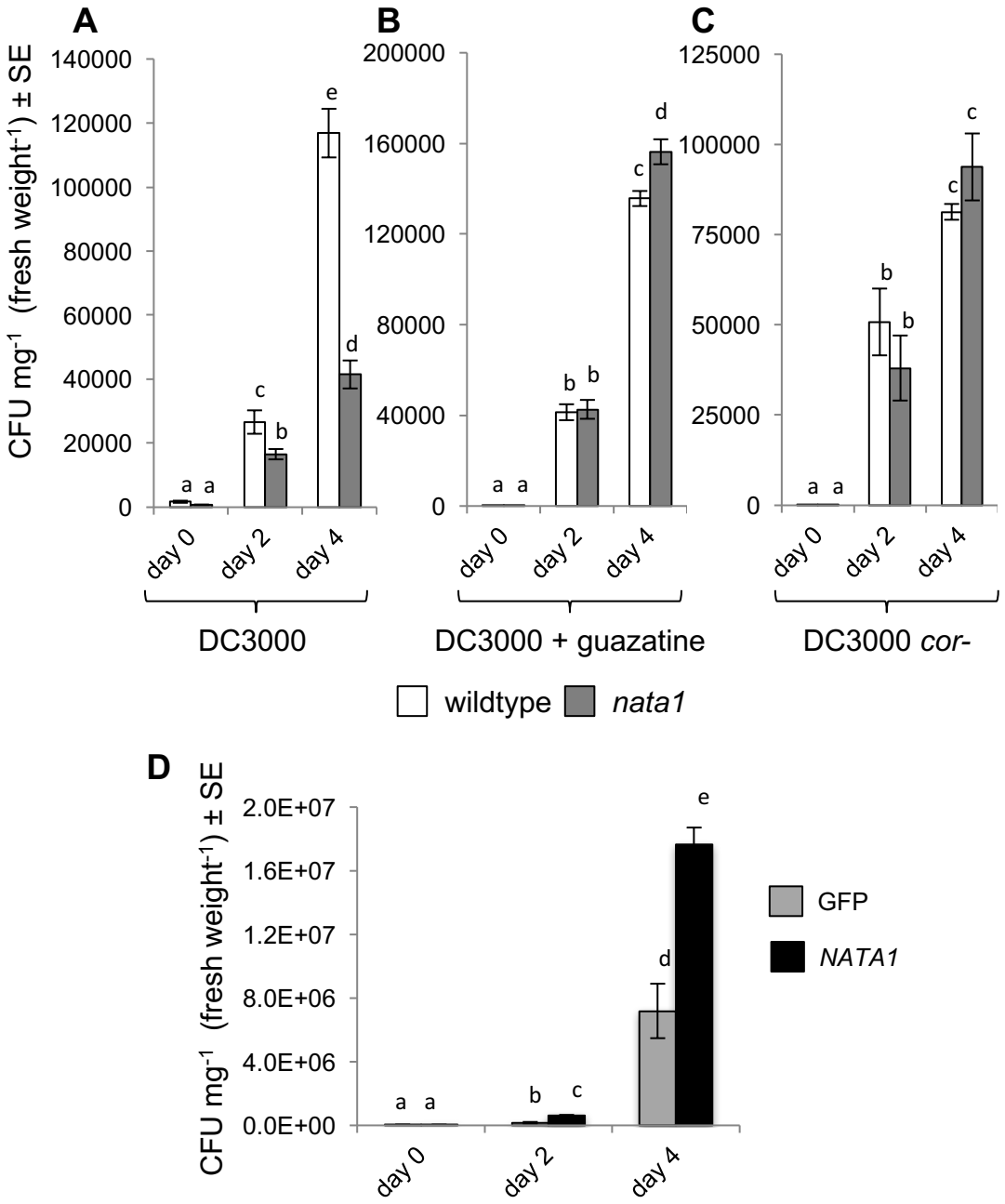


Figure 2.7. Effects of *NATA1* on *P. syringae* growth. Bacterial titers in wildtype Col-0 and *nata1* rosette leaves after infiltrating 10^5 CFU ml^{-1} culture of (A) DC3000, N = 30; (B) DC3000 and guazatine, N = 20; (C) DC3000 (cor-), N = 22-30; different letters indicate significant differences, $P < 0.05$, ANOVA followed by Tukey's HSD test. (D) Growth of *P. syringae* DC3000 *ΔhrcQ-U* in *N. benthamiana* was measured over four days after infiltrating 10^8 CFU ml^{-1} into leaves transiently expressing *NATA1* or *GFP* (control) genes from the constitutive cauliflower mosaic virus 35S promoter. N = 20-30. CFU = colony-forming units. Different letters indicate significant differences, $P < 0.05$, ANOVA followed by Tukey's HSD test.

Therefore, to trigger a virulent infection that is comparable to the Arabidopsis experiments, a DC3000 *ΔhrcQ-U* mutant (Badel *et al*., 2006) was used to infect *N. benthamiana.* Consistent with the Arabidopsis results, DC3000 *ΔhrcQ-U* grew to a higher titer two and four days after infiltration into *N. benthamiana* that was transiently expressing *NATA1* compared to control plants expressing a *GFP* gene (Fig. 2.7D).

Differential responses to *P. syringae* in wildtype and *nata1* Arabidopsis

Reduced *P. syringae* growth in *nata1* mutant Arabidopsis could be due to the induction of other defense responses. Therefore, quantitative RT-PCR assays were used to determine whether known defense-related genes are differentially regulated by *P. syringae* infection of wildtype and *nata1* Arabidopsis leaves. The expression of *PR1*, *PR2* and *PR5,* is commonly used as a marker for the induction of antimicrobial defense responses in Arabidopsis. Consistent with the elevated *P. syringae* resistance of *nata1* plants (Fig. 2.7A), *P. syringae* infiltration induced *PR1* expression to a significantly higher level than in wildtype Arabidopsis (Fig. 2.8A). Induced *PR2* and *PR5* expression showed a delayed response, but ultimately also reached a higher level in *nata1* (Fig. 2.8B and 2.8E). On the other hand, expression of *PR3*, *PR4* and *PR6*, common marker genes for jasmonate-induced plant defenses, was up-regulated to comparable levels in *nata1* and wildtype Arabidopsis leaves (Fig. 2.8C, 2.8D and 2.8F).

As H_2O_2 acts synergistically with salicylic acid to induce plant defense responses (Neuenschwander *et al*., 1995; Sharma *et al*., 1996; Alvarez *et al*., 1998; Mukherjee *et al*., 2010), we measured salicylic acid concentrations in response to DC3000 infection. Although *P. syringae* infiltration into Arabidopsis leaves significantly increased the concentration of salicylic acid two days later, there was no significant difference in free or total salicylic acid content of wildtype and *nata1* leaves (Fig. 2.9A, Supplemental Fig. S8). Similarly, although there was a jasmonic acid increase in *P. syringae*-infected *nata1* leaves relative to controls on day 4 after infection, this was not significantly higher than in *P. syringae*-infected wildtype Arabidopsis (Fig. 2.9B).

Discussion

Our observation of increased H_2O_2 accumulation and *P. syringae* resistance in the Arabidopsis *nata1*mutant is consistent with prior observations of greater pathogen resistance in plants with

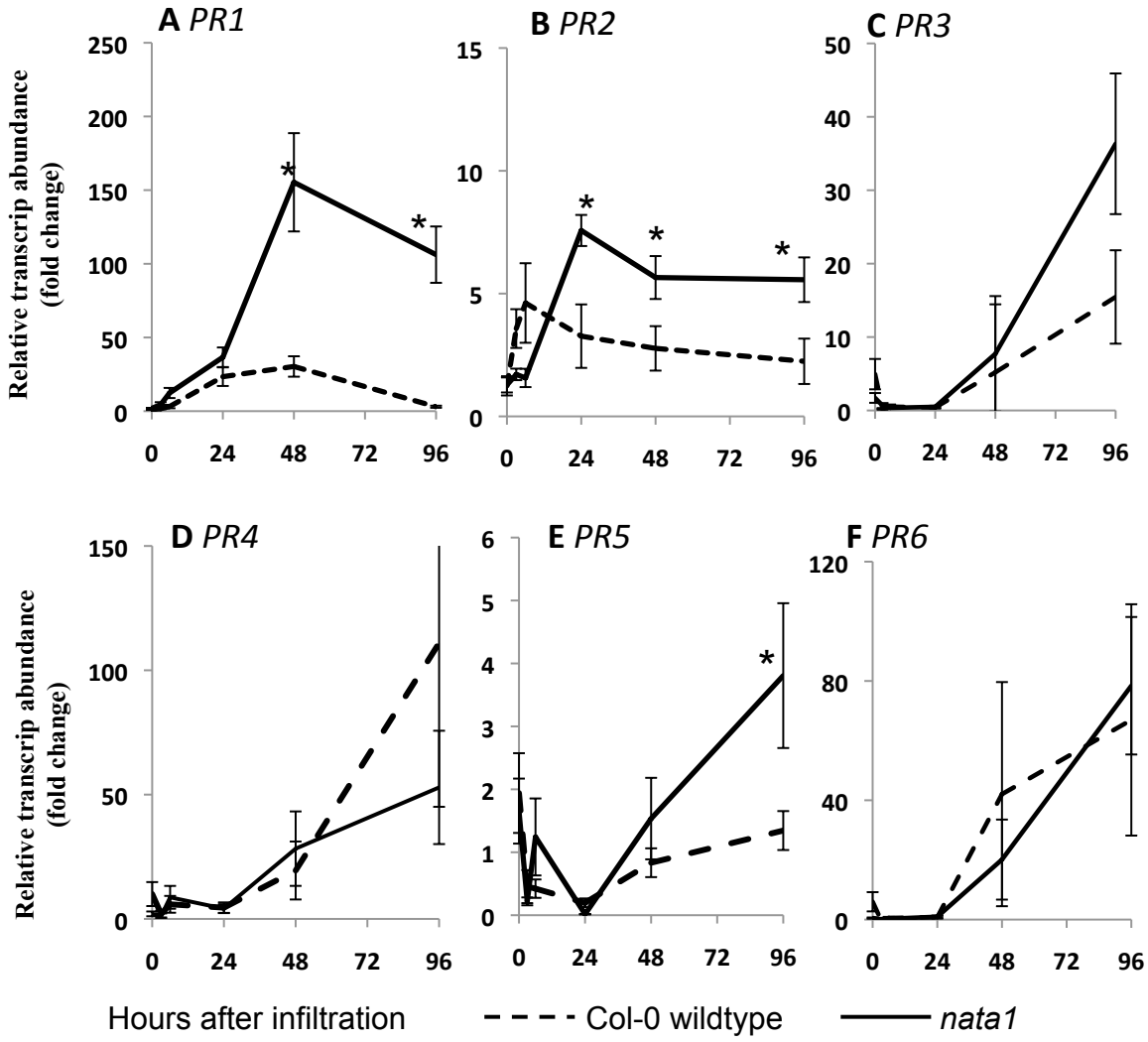


Figure 2.8. Defense-related gene expression in response to *P. syringae*. Wildtype Col-0 and *nata1* were infiltrated with *P. syringae* DC3000. Expression of (A) *PR1*, (B) *PR2*, (C) *PR3*, (D) *PR4*, (E) *PR5* and (F) *PR6* measured by quantitative PCR. Mean ± SE of n = 9. *P < 0.05, two-tailed t-test comparing Col-0 wildtype and *nata1* at the same time point.

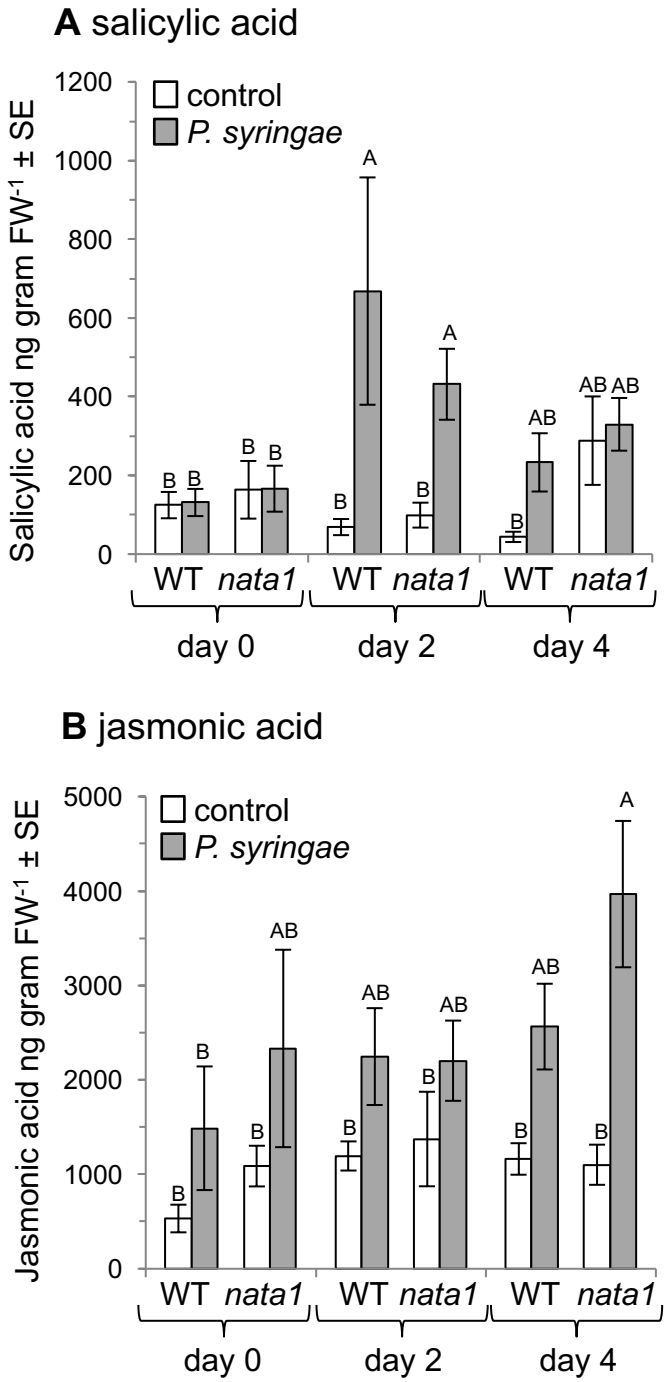


Figure 2.9. Plant hormone induction by *P. syringae* infection. (A) Salicylic acid and (B) jasmonic acid levels at 0, 2, and 4 days after *P. syringae* infiltration into wildtype Col-0 wildtype and *nata1* Arabidopsis leaves. Mean ± SE of N = 12. Within each figure panel, different letters indicate significant differences, $P < 0.05$, ANOVA followed by Tukey's HSD.

elevated polyamines. Overexpression of *S*-adenosylmethionine decarboxylase (*SAMDC1*), which is required to convert putrescine to spermidine, increased spermidine abundance in Arabidopsis (Marco *et al*., 2014). These transgenic plants also had elevated defense gene expression (including *PR1*and *PR2*) and are more resistant to *P. syringae.* Similarly, both spermine synthase overexpression and exogenous addition of spermine made Arabidopsis more resistant to *Pseudomonas viridiflava* (Gonzalez *et al*., 2011). Concomitant addition of a polyamine oxidase inhibitor partly suppressed the plant-protective effect of exogenous spermine, indicating that this oxidation contributes to increased plant resistance.

Induction of NATA1 activity by the *P. syringae* DC3000 coronatine effector results in greater putrescine acetylation and reduced levels of non-acetylated polyamines (Fig. 2.3A and Fig. 2.4). In *nata1* mutant Arabidopsis, there is increased H_2O_2 accumulation from spermine and/or spermidine oxidation (Fig. 2.5). *PR1*, *PR2*, and *PR5* gene expression, which is induced as part of the antimicrobial defense response in Arabidopsis, is increased in *nata1* mutant plants relative to wildtype (Fig. 2.8). As we did not observe a significant difference in the abundance of salicylic acid (Fig. 2.9), defense gene expression may be regulated more directly by changes in H_2O_2 abundance.

A previous report showed a 30% increase in salicylic acid after infection with coronatine-deficient *P. syringae* compared to wildtype *P. syringae* strain *Psm* ES4326 (Zheng *et al*., 2012). However, we observed no salicylic acid differences between wildtype and *nata1* mutant plants (Fig. 2.9A), suggesting that the decrease of salicylate abundance in response to *P. syringae*-derived coronatine is regulated independently of polyamine acetylation. Another recent publication showed that, in the secondary stage of the biotrophic *Plasmodiophora brassicae* (clubroot) infection, enhanced accumulation of salicylate, as well as down-regulated jasmonate pathway induction, was observed in Bur-0, an Arabidopsis ecotype that has a deletion in *NATA1* promoter region and start codon (http://1001genomes.org/; Supplemental Fig. S2.4), but not in Col-0 (Lemarie *et al*., 2015). The role of NATA1 in the observed differences is further indicated by the fact that both *nata1* mutant Arabidopsis and Bur-0 show enhanced resistance to clubroot compared to Col-0 (Lemarie *et al*., 2015). Together with our findings, this may suggest that *NATA1*-mediated polyamine acetylation plays a function in the crosstalk between salicylate and jasmonate defense signaling in a rather complex manner.

Our results are consistent with the hypothesis that NATA1-mediated regulation of H_2O_2 production by PAOs influences plant defense. However, other NATA1 functions may also contribute to the observed phenotypes. For instance, acetylation of diaminopropane by NATA1, which counteracts abscisic acid-dependent stomatal closure (Jammes *et al*., 2014), may have additional effects in plant defense. Moreover, compared to wildtype plants, *nata1* mutants show a smaller attenuation of primary metabolism, particularly sugars and amino acids, after jasmonate treatment (Adio *et al*., 2011). This alteration in primary metabolism could have as yet unknown effects during Arabidopsis defense against *P. syringae* and other pathogens.

The involvement of diverse metabolic and signaling pathways likely accounts for the fact that coronatine is such an effective suppressor of plant defenses during *P. syringae* infection. In addition to promoting stomatal opening and reducing salicylic acid levels (Zheng *et al*., 2012), coronatine also regulates glucosinolate accumulation and thereby inhibits callose induction by indole glucosinolate breakdown (Geng *et al*., 2012). Coronatine-mediated suppression of innate immune defenses in Arabidopsis roots occurs in a COI1-dependent manner, but does not involve direct interference with salicylic acid signaling (Millet *et al*., 2010). Future research will determine whether NATA1 acts in parallel or sequentially with these other coronatine-regulated plant defense pathways.

NATA1-mediated polyamine acetylation induced by *P. syringae*-derived coronatine likely represents the miss-direction of a plant defense response. A possible endogenous plant function for jasmonate-induced *NATA1* expression may be to attenuate H_2O_2 production during insect feeding, which typically triggers a strong jasmonate response in Arabidopsis. N^{δ}-acetylornithine, a metabolic product of NATA1, also has been showed to have direct effects on aphid resistance (Adio *et al*., 2011). The observation that there are at least three Arabidopsis ecotypes that lack NATA1 (Bur-0, Amel-1, and Cal-1) suggests divergent selection for this enzymatic activity. In certain environments the avoidance of coronatine-mediated suppression of Arabidopsis defenses may outweigh any benefits that are provided by NATA1 enzymatic activity.

Compared to the circadian regulation of jasmonate accumulation (Goodspeed *et al*., 2012; Shin *et al.*, 2012), the observed diurnal *N*-acetylputrescine oscillation is slightly delayed, consistent with the observation that *N*-acetylputrescine accumulation is jasmonate-regulated. Several studies have demonstrated circadian regulation of Arabidopsis susceptibility to pathogen

infection (Shin *et al*., 2012; Zhang *et al*., 2013; Ingle and Roden, 2014; Zhou *et al*., 2015). Thus, the circadian cycle of *N*-acetylputrescine biosynthesis (Fig. 2.2C) may be a component of a network of plant responses that contribute to diurnal variation in susceptibility to *P. syringae* infection. In particular, based on the results that we have presented, low constitutive NATA1 activity early in the morning would be expected to promote resistance to virulent *P. syringae* by increasing the availability of polyamines for the production of H_2O_2.

It is likely that polyamine-mediated defense regulation is not limited to Arabidopsis, but rather is more widespread in the plant kingdom. Negative cross-talk between the jasmonate- and salicylate-dependent signaling pathways apparently pre-dates the development of vascular plants (Thaler *et al*., 2002). Similarly, polyamines and their acetylated versions have an ancient evolutionary origin, being present in plants, animals, and microorganisms. Other plant species for which significant amounts of genome data are available contain genes that are predicted to be polyamine acetyltransferases (Supplemental Fig. S2.2), and expression of Arabidopsis *NATA1* in *N. benthamiana* causes not only acetylputrescine accumulation (Fig. 2.2A), but also improved *P. syringae* growth (Fig. 2.7B). To date, acetylated polyamines have been reported in only a relatively limited number of plant species (Deagazio *et al*., 1995; Mesnard *et al*., 2000; Dufeu *et al*., 2003; Fliniaux *et al*., 2004; Kamada-Nobusada *et al*., 2008; Hennion *et al*., 2012; Kim *et al*., 2014). However, this is more likely due to the fact that that these metabolites have not been actively searched for, rather than that they are not present.

Whereas most research on cross-talk between plant defense signaling pathways has focused on the role of transcription factors, the pathway described here is mediated by metabolic enzymes and small molecules. Thus, jasmonate-regulated putrescine acetylation may represent a mechanism whereby H_2O_2 production by polyamine oxidases and the initiation of other antimicrobial plant defense responses can be attenuated. The observation of polyamine-mediated cross-talk between plant defense signaling pathways in Arabidopsis, as well as the evolutionarily ancient origins of the contributing metabolites, suggest that further research is warranted to determine whether similar defense regulation occurs more broadly in other plant species.

Materials and Methods

Plants and growth conditions

Seeds of *Arabidopsis thaliana* ecotypes Ameland-1 (Amel-1), Burren-0 (Bur-0), Calver-0 (Cal-0), Columbia-0 (Col-0), Pedriza (Ped-0), TDr-1, Zal-1, as well as the *nata1* mutation in the Col-0 background (GK-256F07) (Rosso *et al*., 2003) and *nata2* mutation in the Col-0 background, were obtained from the Arabidopsis Biological Resource Center (www.arabidopsis.org). Plants were grown in Cornell mix (by weight 56% peat moss, 35% vermiculite, 4% lime, 4% Osmocoat slow-release fertilizer [Scotts, Marysville, OH], and 1% Unimix [Scotts]) in 20x40-cm nursery flats in Conviron growth chambers with a photosynthetic photon flux density of 200 mmol m^{-2} s^{-1} and a 16:8 h day:night photoperiod, with lights being turned on at 7 AM, at 23°C with a 50% relative humidity.

Methyl jasmonate and coronatine induction

For methyl jasmonate induction, the leaves of 4-week-old Arabidopsis plants were sprayed with an aqueous solution containing 0.01% (v/v) Tween 20, and 0.45 mM methyl jasmonate (diluted from a 1.5 M stock in acetone). Control plants were treated with water containing 0.01% Tween 20 and 0.03% acetone. Plants were covered with plastic domes and leaves were harvested 4 days after elicitation and immediately frozen in liquid nitrogen. Pulverized tissue was extracted and polyamines were analyzed as described below. For coronatine induction, similar assays were performed, with the sprayed aqueous solution containing 0.01% (v/v) Tween 20 and 10 μM coronatine.

Transient gene expression

For transient expression in *N. benthamiana*, *NATA1* and *NATA2* were cloned behind the cauliflower mosaic virus 35S promoter in the T-DNA binary vector pMDC32 as described by Adio *et al*. (2011) and transformed into *Agrobacterium tumefaciens* strain GV3101 for Arabidopsis transformation (Clough and Bent, 1998). A pMDC32::GFP vector, which was generated by cloning GFP amplified from the plasmid pMDC83 (Curtis and Grossniklaus, 2003) as the gene of interest in the same construct, was used as a control, together with an empty vector control. Primers used for cloning are listed in Supplementary Table S1. GV3101 carrying the transgenic constructs was cultured overnight at 30°C in LB broth supplemented with 100 mg ml^{-1} kanamycin. Bacteria were pelleted at 3,600 x g for 20 min, washed three times with sterile water, and resuspended in sterile 10 mM $MgCl_2$ solution to ~0.2 x 10^8 colony-forming units/ml. To reduce gene expression silencing, cultures were mixed with *A. tumefaciens* (0.1 x 10^8 CFU ml^{-1}) transformed with the turnip crinkle virus capsid protein (P38) (Thomas *et al*., 2003). The

bacterial solution was infiltrate into *N. benthamiana* leaves using a 1 ml needle-less syringe. Leaf plugs (8-mm diameter) were collected three days after infiltration to confirm expression of the transgenes by qRT-PCR and four days after infiltration to extract polyamines for HPLC analysis.

Protein extraction and *in vitro* assays

For protein purification, *E. coli* strain M15 was transformed with full-length *NATA1* cDNA cloned with a histidine tag in pQE30 (Qiagen, Hilden, Germany). Expression of recombinant *NATA1* was induced by 0.5 mM isopropyl-β-D-1-thiogalactopyranoside at 22°C for 8 h. Cells were centrifuged at 3000 x g and 4°C for 10 min and resuspended in 0.5 M NaCl, 50 mM NaH_2PO_4 (pH 8.0) prior to being incubated with lysozyme on ice for 30 min. Cells were further disrupted by sonication and centrifuged at 15000 x g for 15 min at 4°C to obtain a clear supernatant containing the soluble proteins. Recombinant NATA1 was purified with the Ni-NTA Purification System from Invitrogen (Carlsbad, California). The supernatants were applied to Ni^{2+}-charged resin equilibrated in extraction buffer. After 60 min of binding with gentle agitation, the resin was washed 3 times with 0.5 M NaCl, 50 mM NaH_2PO_4 (pH 8.0) with 20 mM imidazole, followed protein elution with the same buffer containing increased imidazole (250 mM). After purification, a single protein band of about 27 kD could be observed by SDS-polyacrylamide gel electrophoresis with silver staining. Enzymatic activity toward potential substrates and the catalytic parameter K_m were determined using 2 µg purified protein under the following conditions: 100 mM Tris-HCl (pH 7.5), 2 mM EDTA (pH 7.5) and 10% glycerol at 30°C, with substrate concentration varying from 1.25 to 1000 µM. Ornithine, putrescine, spermidine, spermine, and 1,3-diaminepropane were used as substrates, while acetyl-CoA was used as the acetyl-group donor, as it is the case for known SSATs (Ignatenko *et al*., 1996). Two mM 5-5'-dithiobis[2-nitrobenzoic acid] (DNTB) was added at the end of each reaction to allow spectrophotometric measurement of free CoA generated. Samples were further derivitazed and analyzed by HPLC as described below for confirmation of the expected products. K_m values were determined from Michaelis–Menten plots, and non-linear least-squares fitting of data was performed using Microsoft Excel with Solver add-in software (www.solver.com).

Quantification of free polyamines

Polyamines were extracted and derivatized either with either benzoyl chloride or 6-aminoquinolyl-N-hydroxysuccinimidylcarbamate (AQC) using an AccQ-Fluor reagent kit (Waters) prior to HPLC detection. One hundred mg of fresh rosette leaves were ground in liquid

nitrogen to fine powder with 3 mm steel beads using a Harbil model 5G-HD paint shaker (Fluid Management, Wheeling, IL). Ground tissue was taken up in 20 mM HCl (10 ml mg^{-1} of leaf tissue) containing 40 µM *L*-norleucine as an internal standard. Extracts were centrifuged at 15,000 x g at 23°C for 20 min and the supernatants were saved for analysis. For derivatization, 5 µl extracts were mixed with 35 µl borate buffer prior to initiating the reaction by adding 10 µl AQC solution. The mixture is then immediately incubated at 55°C for 10 min and 10 µl aliquots of each sample were injected onto a Nova-Pak C18 column (3.9x150 mm particle size 4µm) using a Waters 2695 pump system. A Waters model 2475 fluorescence detector with an excitation wavelength of 250 nm and an emission wavelength of 395 nm was used to detect eluted amino acid derivatives, with the data being recorded by Waters Empower Software. Solvent A (containing sodium acetate and tris-acetate- ethylenediaminetetraacetic acid (TAE) at pH 5.05) was purchased premixed from Waters; Solvent B was acetonitrile:water (60:40). The gradient used was 0 to 0.01 min, 100% A; 0.01 to 0.5 min, linear gradient to 3% B; 0.5 to 12 min, linear gradient to 5% B; 12 to 15 min, linear gradient to 8% B; 15 to 40 min, linear gradient to 30% B; 40 to 43 min, linear gradient to 37% B; 43 to 46 min, linear gradient to 42% B; 46 to 54 min, linear gradient to 45% B; 54 to 58 min, linear gradient to 50% B; 58 to 60 min, linear gradient to 60% B; 60 to 67 min, linear gradient to 80% B; 68 to 71 min, linear gradient to 100% B. The flow rate was 1.0 mL min^{-1}.

For the benzoylation method, polyamines were extracted from the ground leaf powder in water with 1% HCl containing 25 µM 1,6-hexanediamine (internal standard), derivatized, and detected by HPLC (Yoda *et al*., 2003). The extracts were centrifuged at 15,000 x g at 10°C for 20 min. Five hundred µl of the supernatants were transferred to fresh tubes, 500 µl 2 N NaOH and 10 µl benzoyl chloride were added, and samples were incubated at 48°C for 20 min. Following incubation, 1 ml saturated NaCl was added, samples were mixed, and benzoylated polyamines were extracted by the addition of 1 ml diethyl ether after centrifuging the extract at 5,000 x g at 25°C for 10 min. The upper phase was collected and evaporated under N_2 gas. After complete evaporation, the residue was dissolved in 200 µl diethyl ether and evaporated under N_2 gas. Benzoylated polyamines were dissolved in 50 µl of 50% acetonitrile for HPLC analysis with the system described above. The gradient was water (solvent A) and 100% acetonitrile (solvent B), with a flow rate of 1.0 mL min^{-1} at 28°C with the following gradient: 0-1 min 100% A; 1-3 min 85% A, 3-10 min 70% A,10-20 min 47.5% A, 20-21 min 100 % B; 21-27 min 100% B; 27-

28 min 100% A; 28-35 min 100% A. Separated benzoylated polyamines were detected at 229 nm with Waters 2996 photodiode array (PDA) detector. Known concentrations of polyamine standards were treated according to both procedures described above and standard curves were used for polyamine quantification.

Bacterial growth assays

Pseudomonas syringae strains DC3000 and DC3000 Cor- [mutant line DB29 cfa- cma- (Millet *et al*., 2010)] were supplied by N. Clay (Yale University, New Haven, CT). DC3000 *ΔhrcQ-U* was supplied by A. Collmer (Cornell University, Ithaca, NY). Bacteria were cultured at 30°C in LB (Luria-Bertani) broth supplemented with 50 μg ml^{-1} rifampicin and 50 μg ml^{-1} kanamycin. For leaf infiltration, bacterial cultures were centrifuged at 3000 x g for 10 min, resuspended in water, and diluted to approximately 10^5 colony forming units (CFU) ml^{-1}. Three-week-old Col-0 wild-type and *nata1*leaves were pressure-infiltrated using a 1-ml syringe and excess bacterial solution was wiped off with a paper towel. Plants were infiltrated in a staggered manner and leaf discs (8-mm diameter) were collected on the same day, at 0, 2, and 4 d after infiltration and submerged in 1 ml sterile water with 0.01% Tween 20 for 4 h to allow equilibration of the bacteria between the apoplastic space of the leaf disks and the surrounding water, as described previously (Anderson *et al*., 2006). Ten μl samples of a dilution series (10^{-1} to 10^{-6}) prepared in 96-well microtiter plates were spotted on an LB agar plate supplemented with 50 μg ml^{-1} rifampicin and incubated at 30°C. After 2 d, bacterial colonies were counted at a point in the dilution series where there were fewer than about 50 colonies in the spotted cluster, and the concentration of bacteria relative to the weight of the leaf disks was calculated.

For bacterial growth in *N. benthamiana*, DC3000 ΔhrcQ-U was cultured at 30°C in LB broth supplemented with 50 μg mL^{-1} rifampicin and 50 μg ml^{-1} kanamycin. Overnight bacterial cultures were centrifuged at 3000 x g for 10 min, resuspended in water, and diluted to approximately10^8 CFU ml^{-1}. *Nicotiana benthamiana* leaves were infiltrated with *P. syringae* suspension two days after they had been infiltrated with *A. tumefaciens* carrying the desired overexpression construct. *Pseudomonas syringae* growth in *N. benthamiana* leaves was measured as described above for Arabidopsis.

H_2O_2 Detection and quantification

H_2O_2 was detected and quantified by 3.3'-diaminobenzidine (DAB) staining (Ramel *et al*., 2009; Daudi *et al*., 2012). Leaves from mock- or *P. syringae*-infiltrated plants were placed in 12-well

microtiter plates. Two ml of 1mg ml^{-1} DAB solution with 0.05 % v/v Tween 20 (pH 3.0) were pipetted into the wells, plates were covered with aluminum foil, and were placed on a platform shaker at 100 rpm for 4 h at 25°C. After incubation, the DAB solution was replaced with 2 ml of bleaching solution (3:1:1 ethanol: acetic acid: glycerol) and plates were placed in water bath at 85-90°C for 15 to 20 min. Following bleaching, the solution in the wells was replaced with 1 ml of fresh bleaching solution and leaves were used for photography or quantification. For quantification assays, DAB-stained leaves were ground in liquid nitrogen and homogenized in 0.2 M $HClO_4$. Following homogenization, samples were centrifuged at 12,000 x g for 10 min at 4°C. Supernatants were transferred to new tubes and their absorbance was measured at 450 nm. Quantification of H_2O_2 was calculated by comparing the absorbance values with a standard curve prepared with known H_2O_2 concentrations (5 to 200 μmol ml^{-1}).

Gene expression analysis

Relative transcript abundances of *PR1* (At2g14610), *PR2* (At3g57260), *PR3* (At3G54420), *PR4* (At3G04720), *PR5* (At1G75040), *PR6* (At2G38900), *PAO1* (At5g13700), *PAO2* (At2g43020), *PAO3* (At3g59050), *PAO4* (At1g65840), *PAO5* (At4g29720), *SPDS1* (AT1G23820), *SPDS2* (AT1G70310), *SPMS* (AT5G53120) and *NATA1* (At2g39030) were analyzed and compared by qRT-PCR using elongation factor 1-α (EF1α, At5g60390) as the internal standard. Leaves (~30 mg) from sample groups were ground in liquid nitrogen and total RNA was extracted using the SV total RNA isolation system (Promega, www.promega.com) with on-column DNAse treatment. After the verification of the integrity of RNA and quantification with a Nanodrop system, 1 μg of total RNA was reverse transcribed using AMV Reverse Transcriptase (Promega) and RNasin Ribonuclease inhibitor (Promega) using oligo$(dT)_{15}$ as primer. Following cDNA synthesis, the samples were diluted 10-fold in nuclease free water. Gene-specific primers (Supplementary Table S2) were designed using Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). qRT-PCR reactions were performed using a Applied Biosystems' 7900HT Instrument with the SYBR Green PCR master mix (Applied Biosystems, www.appliedbiosystems.com). The reaction mixture (10 μL total volume) contained 3.3 μL of cDNA solution and 6.7 μL PCR master mix (5 μL of SYBR Green master mix, 0.4 μL of forward and reverse gene specific primers (both at 10 μM) and 0.9 μL nuclease free H_2O). Reactions were initiated by the activation of the enzyme at 95°C for 10 min, followed by 40 cycles of: 95°C for 15 s, 60°C for 15 s and 72°C for 15 s and final extension at 72°C for 2 min.

The resulting C_T values were used to calculate the relative transcript abundance according to the standard curve method.

Polyamine oxidase (POA) enzyme activity assay

POA activity was assayed spectrophotometrically (Lim *et al*., 2006; Tavladoraki *et al*., 2006). Leaves (200 mg) were ground in liquid nitrogen, homogenized in 0.2 M sodium phosphate buffer pH 6.5 (1:5 w/v), and centrifuged and at 12,000 x g for 20 min. Supernatants were moved to fresh tubes and half of the total volume was used for protein quantification by Bradford assay using bovine serum albumin as the standard (Bradford, 1976). The reaction mixture contained 1 mg ml^{-1} horseradish peroxidase, 0.1 mM 4-aminoantipyrine, 1 mM 3,5-dicholor-2-hyrodxybenzesulphonic acid, and 2 mM spermine in 0.2 M Na-phosphate buffer (pH 6.5). The reaction was initiated by the addition of 100 µl of the leaf homogenate into the reaction mixture and absorbance at 515 nm was recorded after incubating for 20 min at 25°C. Specific enzyme activity was calculated per mg protein.

Guazatine inhibition assays

For PAO inhibition treatments, 1 mM guazatine acetate solution containing 2.5 % Tween 20 was sprayed onto plants 2 days before *P. syringae* infiltration (Fu *et al*., 2011). The guazatine concentration in the leaves was determined by HPLC and quantified using a standard curve based on samples with known guazatine concentrations. As guazatine is a mixture of compounds, the two most abundant peaks in the HPLC runs of guazatine solution were used for quantification, preparation of a standard curve, and estimation of leaf guazatine concentrations. Leaf samples (100 mg) were extracted with 20 mM HCl containing 100 pM *L*-norleucine as the internal standard and centrifuged at 12000 x g at 25°C for 15 min. Supernatants were transferred to new tubes and used for derivatization with 6-aminoquinolyl-N-hydroxysuccinimidylcarbamate using an AccQ-Fluor reagent kit (Waters). For derivatization, 5 µl extract was mixed with 35 µl borate buffer and the reaction was initiated by the addition of 10 µl AccQ-Fluor reagent (Waters, Millford, MA), followed by mixing and incubation for10 min at 55ºC. Ten microliters of each sample were injected onto a Nova-Pak C18 column (3.9x150 mm particle size 4µm) using a Waters 2695 pump system at 38°C . Eluted guazatine derivatives were detected using a Waters model 2475 fluorescence detector with an excitation wavelength of 250 nm and an emission wavelength of 395 nm, and the data were recorded using Waters Empower software. Solvent A, containing sodium acetate and TAE at pH 5.05, was purchased premixed from Waters; Solvent B

was acetonitrile:water (60:40). Linear gradient of the run was as follows with a 1.0 mL min^{-1} flow rate: 1-12 min 97% A,; 12-15 min 95% A,; 15-40 min 92% A,; 40-43 min 70% A, B; 43-46 min 63% A,; 46-54 min 58% A,; 54-58 min 55% A,; 58-60 min 50% A,; 60-67 min 40% A;; 67-67.1 min 20% A,; 67.1-71 min 0 %A,; 71-75 min 100 % A.

For *in planta* growth experiments, *P. syringae* DC3000 was cultured at 30°C in LB broth supplemented with 50 mg mL^{-1} rifampicin, centrifuged, resuspended in sterile water, and diluted to a concentration of 10^{-5} CFU ml^{-1}. Bacterial cultures were pressure-infiltrated into leaves with a syringe and samples were collected to measure bacterial growth, as described above. To test the effect of guazatine on *P. syringae* growth in culture, 0, 1, 10 and 100 µM guazatine acetate was added to LB medium, cultures were inoculated with 1:10 diluted (500 µl into 5 ml) fresh cultures, and OD_{600} (optical density at 600 nm) was measured at 0, 1, 3, 6, 9, 12, 24 and 48 h after inoculation.

Phytohormone analysis

For free phytohormone analysis, 100 mg of fresh rosette leaves were ground in liquid nitrogen and extracted in 1 ml of isopropanol:water:HCl (2:1:0.005) buffer. Eighty ng each of D_4-salicylic acid and D_5-jasmonic acid (C/D/N Isotopes Inc., www.cdnisotopes.com/) were added as internal standards. After homogenization in a FastPrep® homogenizer (MP Biomedicals, www.mpbio.com) at 6 m s^{-1} for 45 s, samples were extracted with dichloromethane, dried in a rotary evaporator and dissolved in 200 µL methanol. For free and conjugated salicylic acid measurement, 200 mg of fresh rosette leaves were extracted in 1 ml 90% methanol prior to being re-extracted in 0.5 ml 100% methanol. Sixty ng of D_4-salicylic acid was added as an internal standard. Extracts were combined and dried completely prior to being resuspended in 5% trichloroacetate and extracted with an ethylacetate-cyclopentane-isopropanol (50:49:1) buffer. The organic phase, containing free salicylic acid, was collected, dried under nitrogen flow and dissolved in 200 µL methanol. The aqueous phase was adjusted to pH 1 with concentrated HCl, boiled for 30 min, and extracted again as described above. The resulting organic phase was dried under nitrogen flow and dissolved in 200 µL methanol for total salicylic acid analysis.

Extracted samples were analyzed using a triple quadrupole liquid chromatography-tandem mass spectrometry system (Thermo Scientific, www.thermoscientific.com) on a C18 reverse-phase HPLC column (3 µm, 150 × 2.00 mm; Gemini-NX; Phenomenex, www.phenomenex.com), as described previously (Rasmann *et al*., 2012). Analytes were

separated using a gradient of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) at a flow rate of 300 μL min−1. Phytohormones were analyzed by negative electrospray ionization (spray voltage, 3.5 kV; sheath gas, 15; auxiliary gas, 15; capillary temperature, 350°C), collision-induced dissociation (argon gas pressure, 1.3 mTorr; energy, 16 V), and selected reaction monitoring of compound-specific parent/product ion transitions (salicylic acid, 137→93; D_4-SA, 141→97; jasmonic acid, 209→59; D_5-JA, 214→62).

Sequence Alignment and Phylogenetic Tree Generation

Arabidopsis NATA1 homologs in the Brassicaceae and other plants were obtained by comparison to genomic and EST data in GenBank and Sol Genomics Network. The closest *Homo sapiens* (human) NATA1 homolog of was included as an outgroup. Amino acid sequences were aligned using Clustal Omega (Sievers *et al*., 2011) and rooted phylogeny trees were constructed with MEGA5 using a maximum-likelihood algorithm (Jones-Taylor-Thornton probability model, gamma distributed rates among sites). To evaluate the tree topology, a bootstrap resampling analysis with 1,000 replicates was performed.

Statistical Analysis

Statistical analysis was conducted using JMP software (www.jmp.com).

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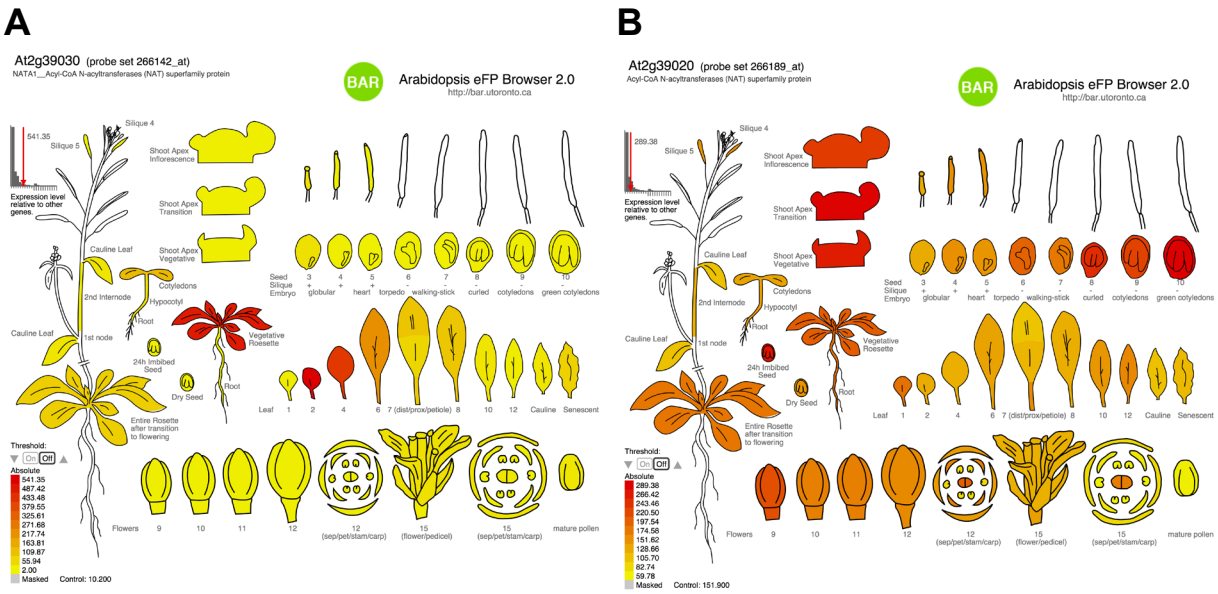
Supplemental Table 2.1. Primers used for cloning and PCR amplification in this study

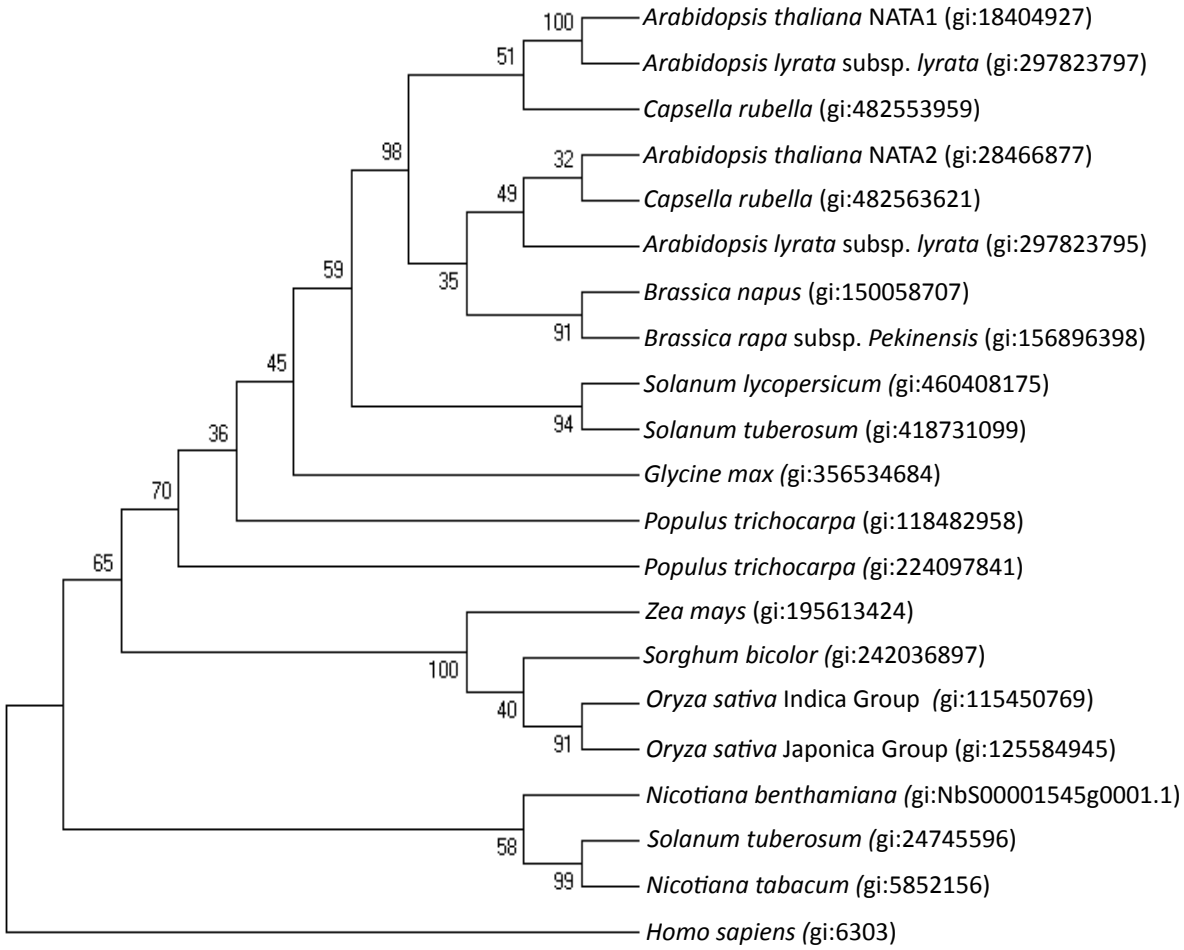
Target Gene		Primer sequence
NATA1 (At2g39030)	forward	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CAT GGC GCC TCC AAC CGC AGC ACC A
	reverse	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC CTA GAT GTT TAG CTT GTC AAT AGC TTG AAG
NATA2 (At2g39020)	forward	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CAT GGC AGC CGC CGC ACC G
	reverse	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC CTA GAT GTT GAC CTG ATC AAA AGC TTC
GFP (from pMDC83)	forward	GGGG ACA AGT TTG TAC AAA AAA GCA GGC TAA ATG AGT AAA GGA GAA GAA CTT TT
	reverse	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTT TAG TGG TGG TGG TGG TG
NATA1 promoter region	forward	TAA GCT AAA GAA TTA AAG TAG TAA
	reverse	GGA TTC TCG AAA CCC TAA TCT TC

Supplemental Table 2.2. Primers used for quantitative RT-PCR

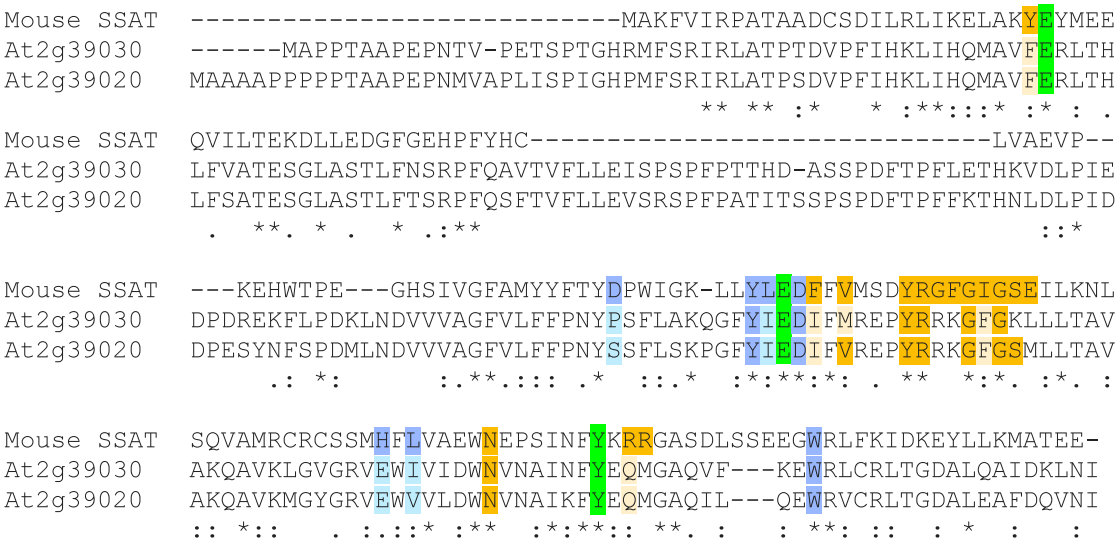
Target Gene		Primer sequence
EF1-α (At5g60390)	forward	TGAGCACGCTCTTCTTGCTTTCA
	reverse	GGTGGTGGCATCCATCTTGTTACA
PR1 (At2g14610)	forward	TGATCCTCGTGGGAATTATGT
	reverse	TGCATGATCACATCATTACTTCAT
PR2 (At3g57260)	forward	AGCTTCCTTCTTCAACCACACAGC
	reverse	TGGCAAGGTATCGCCTAGCATC
PR3 (At3G54420)	forward	TCTAGCTTGAACGGTGGCTGTG
	reverse	CGTGCCATTAACGGTGCTTTGG
PR4 (At3G04720)	forward	TCCAAATCCAAGCCTCCGTTGC
	reverse	GCGGCAAGTGTTTAAGGGTGAAG
PR5 (At1G75040)	forward	AGCAATGCCGCTTGTGATGAAC
	reverse	ATCACCCACAGCACAGAGACAC
PR6 (At2G38900)	forward	ATACATGTCTAGCCGGCGGTTG
	reverse	TTGCTACCGCGTTAGGGTTTGG
PAO1 (At5g13700)	forward	GCTCTAACATTCTGGTGGTGACG
	reverse	CCCAAACATGTCCCTGAGAACAC
PAO2 (At2g43020)	forward	AGATTGTAGGATGCGAGTC
	reverse	TTAGACGATATAAGAAGAGG
PAO3 (At3g59050)	forward	ACAAACCTCACGACCTCTATG
	reverse	TCAAGCACACGCATCCTG
PAO4 (At1g65840)	forward	GGGAACAGTGACATTCTCGAAAC
	reverse	AATTGGAACCCTGCTTCTGTCTG
PAO5 (At4g29720)	forward	GATGACCTAGACGCAATG
	reverse	ATGAGTTGTGGAGTAATGG
NATA1 (At2g39030)	forward	AGCAGATGGGTGCGCAGGTT
	reverse	TCGCTCGATGGGTCTCATGCA
SPDS1 (AT1G23820)	forward	GAAGAGGATAACGGCGGC
	reverse	CAGAGAACCACCCAGGAATAACAG
SPDS2 (AT1G70310)	forward	TGATTTGCCCGTGAAGAGACC
	reverse	GAGAACCATCCAGGAATAATAGAGG
SPMS (AT5G53120)	forward	GTTGTTGGTGGAGGTGATGGTG
	reverse	GGATTTACGGAGGAACTCAGCAG



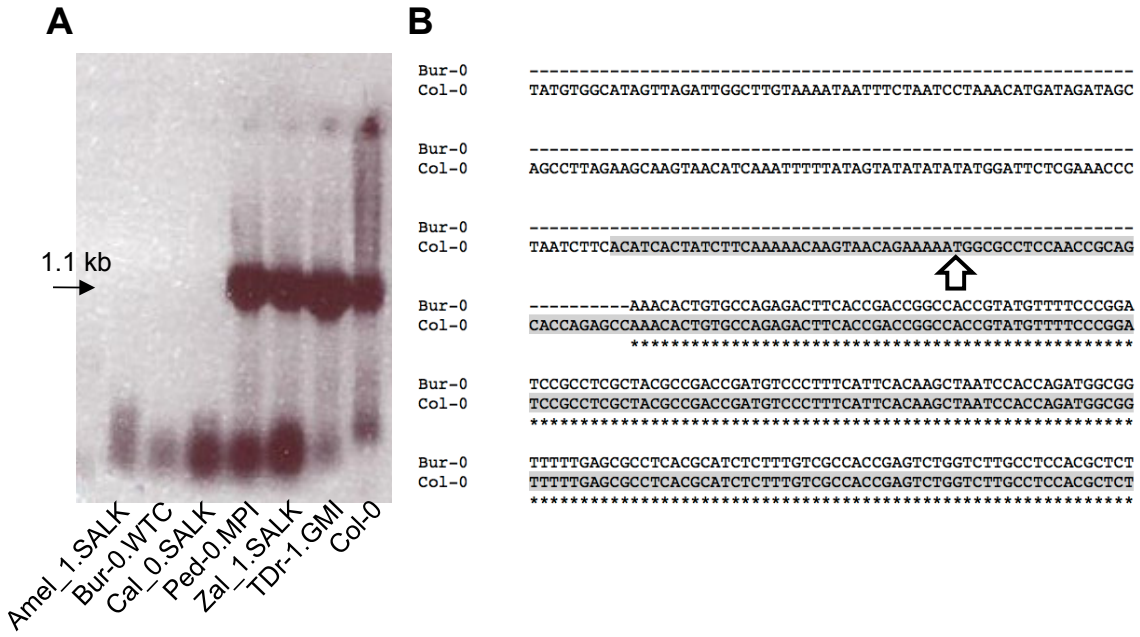
Supplemental Figure S2.1. Spatial and temporal expression of (A) *NATA1* and (B) *NATA2* from Botany Array Resource (http://bar.utoronto.ca/welcome.htm).



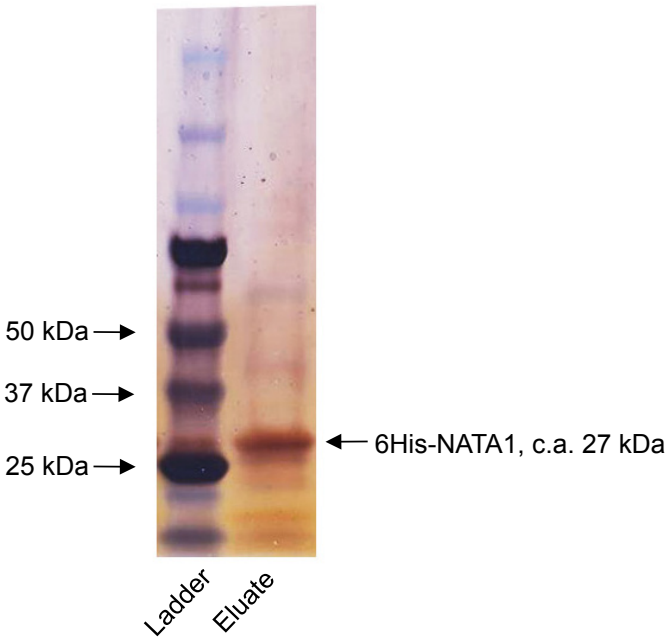
Supplemental Figure S2.2. Phylogenetic tree of plant proteins with similarity to polyamine acetyltransferases. Phylogenetic tree of NATA1 protein homologs. Human (*Homo sapiens*) spermidine/spermine N^1-acetyl-transferase (SSAT) was used as an outgroup. A consensus phylogenetic tree was produced with 1000 replicates. Values at the branch points indicate bootstrap percentages.



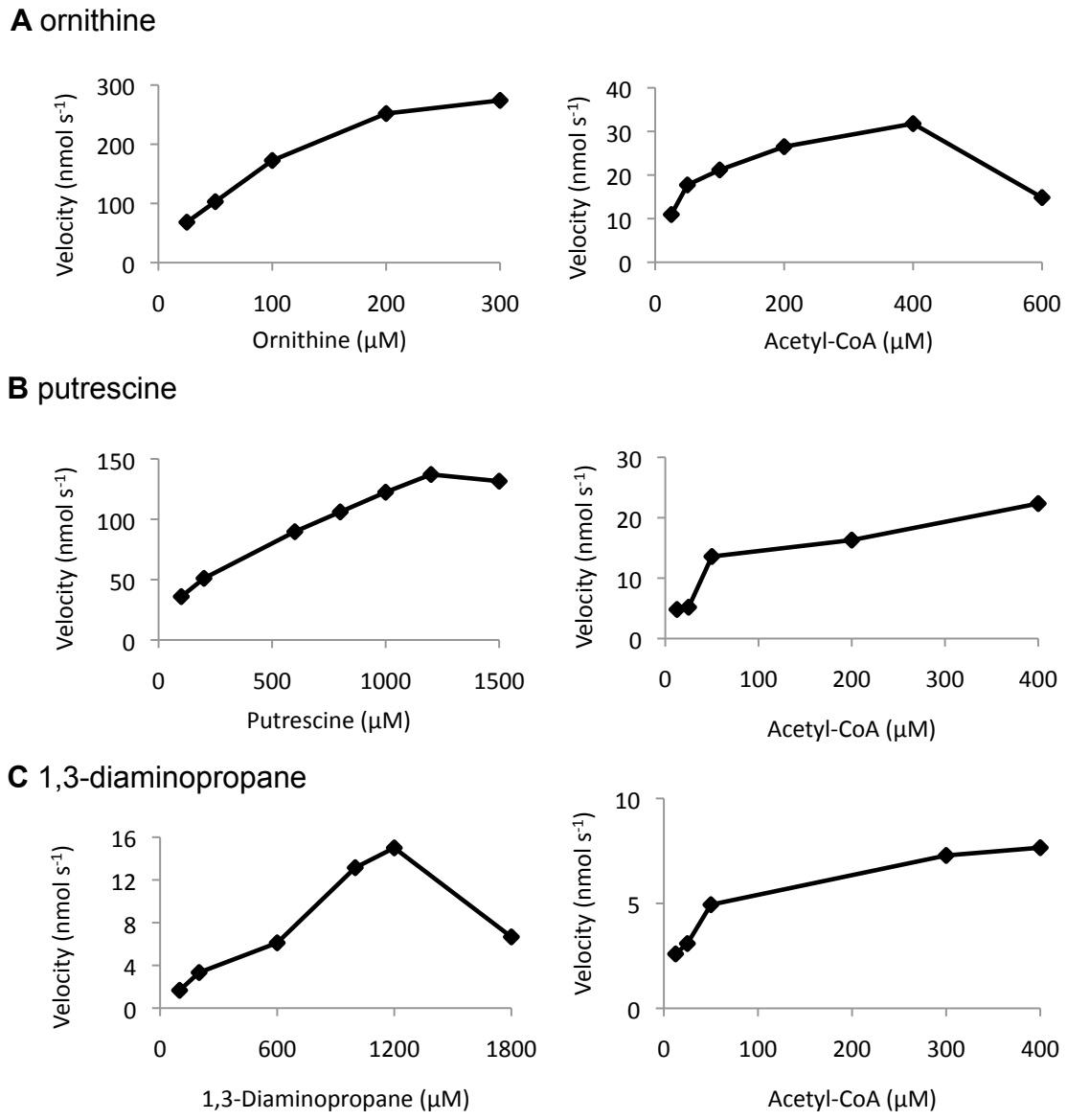
Supplemental Figure S2.3. Active site amino acid sequence alignment of Arabidopsis NATA1 and NATA2 with mouse spermidine/spermine acetyltransferase (SSAT; GI:6677849). Orange: CoA binding region, blue: spermine binding residues, and green: proposed catalytic site. Sequences were aligned with Clustal Omega.



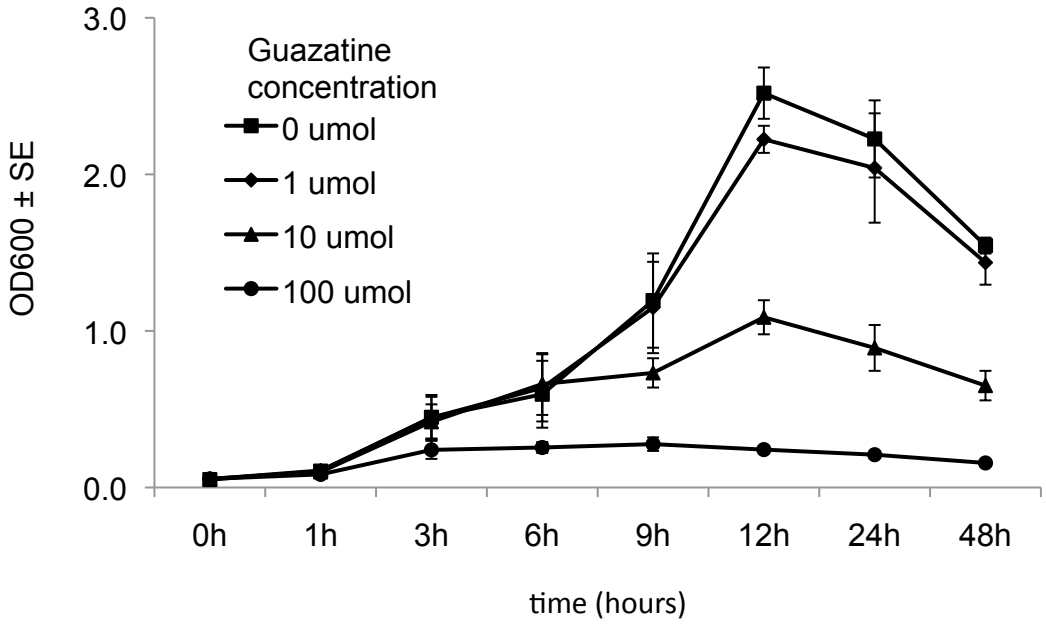
Supplemental Figure S2.4. **Arabidopsis ecotypes with natural *nata1* knockout mutations were identified through 1001 Genomes Projects.** (A) Potential promotor region amplification by PCR. Left to right: Amel_1, Bur-0, Cal_0, Ped-0, Zal_1, TDr-1 and Col-0. (B) Sequence alignment of Chr2:16305177..16305536 from Bur-0 and Col-0. The NATA1 transcript is marked in grey and the ATG start codon is indicated with and arrow. Sequences were aligned with Clustal Omega.



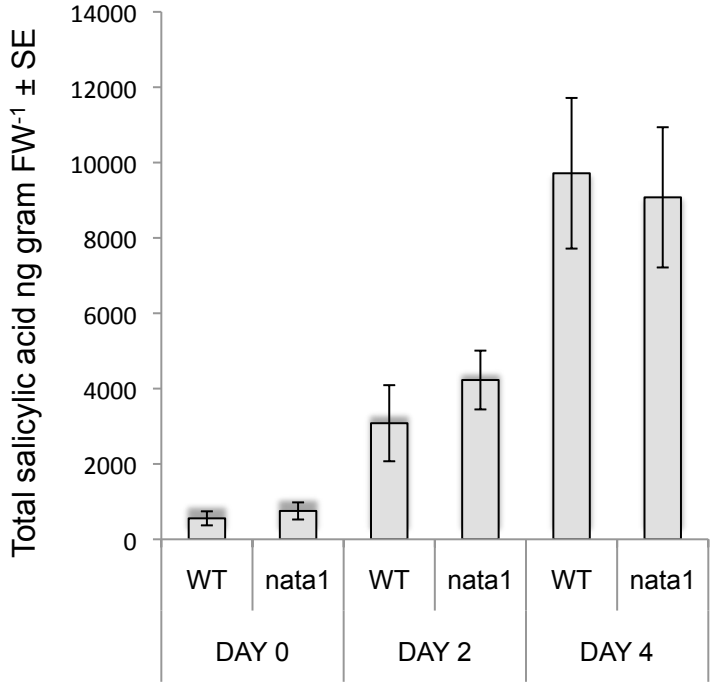
Supplemental Figure S2.5. Heterologously expressed NATA1 were purified with Ni-NTA and confirmed using a silver-stained protein gel. Shown is a representative gel.



Supplemental Figure S2.6. Representative Michaelis Menten curves. The kinetics of heterologously expressed NATA1 were determined by Michaelis-Menten curves for (A) ornithine, (B) putrescine and (C) 1,3-diaminopropane by changing concentration of the potential substrate and acetyl-CoA, respectively. Shown are means of three technical replicates.



Supplemental Figure S2.7. **Growth of *P. syringae* in LB medium supplemented with guazatine.** Mean ± SE of N =4.



Supplementary Figure S2.8. Time course of total salicylic acid accumulation in response to *P. syringae* infection. Mean ± SE of n = 12.

CHAPTER THREE

THE ARABIDOPSIS NATA2 PROTEIN IS A POLYAMINE ACETYLTRANSFERASE THAT CONTRIBUTES TO HEAT STRESS TOLERANCE AND DEFENSE*

Abstract

Polyamines are cationic metabolites that are found in all living organisms and play a central role in numerous aspects of physiology in plants, animals, and microorganisms. The cellular homeostasis of polyamines is tightly controlled to prevent undesired signaling and regulation. Here we provide *in vivo* and *in vitro* evidence that *Arabidopsis thaliana* (Arabidopsis) NATA2, a polyamine acetyltransferase that preferentially uses ornithine and spermine as substrates, contributes to polyamine homeostasis in plants. The upregulation of NATA2 by heat stress results in decreased polyamine abundance in plant tissue, which in turn leads to decreased heat tolerance in Arabidopsis in seedlings. Under high temperature, the NATA2-mediated shift of polyamine titer also causes alteration in plant resistance to the semi-biotrophic pathogen *Pseudomonas syringae.* More specifically, by reducing H_2O_2 formation via polyamine oxidation, NATA2 decreases H_2O_2-dependent defense in Arabidopsis and makes plants more susceptible to *P. syringae.* Moreover, the shift in polyamine abundance affects plant defense against chewing herbivores, as *Spodoptera exigua* caterpillars grow more slowly on *nata2* knockout mutants under heat stress. Together, these results highlight that polyamines play a critical role in plant defense signaling in response to multiple stresses. Two *NATA* homologs, *NATA1* and *NATA2*, are found as a tandem duplication in the Arabidopsis genome. However, an attempt to obtain a double knockout mutant deficient in both *NATA* genes was unsuccessful, suggesting that polyamine acetyltransferases are essential for plant survival. In summary, our results indicate that polyamine acetylation is an alternative molecular mechanism for controlling plant polyamine homeostasis, in addition to biosynthesis, transportation and catabolism.

*Yann-Ru Lou designed and performed most of the experiments. Dr. Yiping Qi generated the double mutant, Dr. Navid Mohaved assisted with mass spectrometry assays and Ms. Hannah Powell assisted with pathogen infection experiments. The article was written with help from Dr. Georg Jander.

Introduction

Polyamines are nitrogen-containing metabolites that are found in all living cells. In plants, the most abundant polyamines are the diamine putrescine, the triamine spermidine, and the tetraamine spermine (Fig. 3.1A). These compounds play a central role in many aspects of plant physiology by influencing transcription, RNA modification, protein synthesis, enzyme activity, and signaling pathways (Evans and Malmberg, 1989; Galston *et al.*, 1997; Kusano *et al.*, 2007; Kusano *et al.*, 2008; Takahashi and Kakehi, 2009; Wimalasekera *et al.*, 2011; Sagor *et al.*, 2015). In most plants, putrescine, the precursor for the synthesis of higher polyamines, is synthesized from arginine in two parallel pathways involving ornithine decarboxylase and arginine decarboxylase, respectively (Figure 3.1B) (Galston and Sawhney, 1990; Bagni and Tassoni, 2001). To form spermidine, an aminopropyl group from decarboxylated S-adenosylmethionine is added to putrescine by spermidine synthase. To form spermine, an additional aminopropyl group from the same source is added to spermidine by spermine synthase (Bagni and Tassoni, 2001).

Plant polyamines are present in free, conjugated, soluble or insoluble forms. Although a role in plant growth and development, including cell division, flowering, cell wall cross-linking and stress responses has been suggested for conjugated polyamines, the physiological functions remain under-investigated (Bouchereau *et al.*, 1999; Bassard *et al.*, 2010; Moschou *et al.*, 2012). Some of the best-studied conjugated forms are phenylpropanoid-polyamine conjugates and hydroxycinnamic acid amides (Fellenberg *et al.*, 2009; Grienenberger *et al.*, 2009; Luo *et al.*, 2009; Kaur *et al.*, 2010; Onkokesung *et al.*, 2012). Acetylated polyamines, on the other hand, have been reported only occasionally in plants (Bagni and Tassoni, 2001; Hennion *et al.*, 2011; Tavladoraki *et al.*, 2012). For instance *N*-acetylputrescine has been found in *Daucus carota*, *Nicotiana plumbaginifolia*, *Datura stramonium*, and *Arabidopsis thaliana* (Arabidopsis) (Mesnard *et al.*, 2000; Fliniaux *et al.*, 2004; Lou *et al.*, 2016). Interestingly, N^1-acetylspermidine is as abundant as spermidine in both roots and above-ground tissue of Arabidopsis (Kamada-Nobusada *et al.*, 2008). However, the metabolic function of acetylated polyamines in Arabidopsis and other plants has not been investigated, and the enzymes involved in their biosynthesis and degradation remain largely unknown.

Despite being widely viewed as accumulative products, acetylated polyamines have been showed to influence metabolism by acting as pathway intermediates, facilitating turnover and translocation of polyamines (Bassard *et al.*, 2010; Moschou *et al.*, 2012; Tavladoraki *et al.*,

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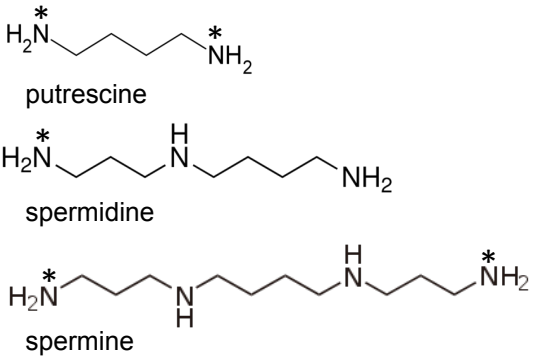

B.

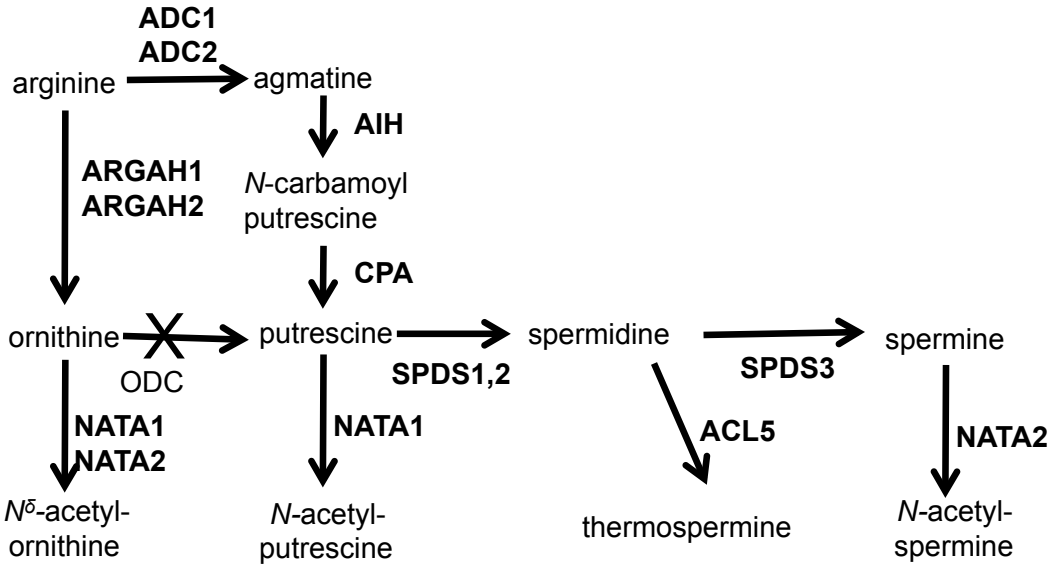

Figure 3.1. (A) Structures of commonly found polyamines. Asterisks indicate their N^1-acetylation sites. (B) Pathways for polyamine synthesis and catabolism in plants. Names of known Arabidopsis proteins catalyzing the reactions are indicated. No gene encoding ornithine decarboxylase has been found in Arabidopsis.

2012). In Arabidopsis, a GNAT family acetyltransferase, NATA1 (N-ACETYLTRANSFERASE ACTIVITY1; At2g39030), which acetylates ornithine and putrescine to produce N^{δ}-acetylornithine and *N*-acetylputrescine, respectively, was recently identified (Adio *et al.*, 2011; Lou *et al.*, 2016). NATA1 competes with higher-order polyamine formation and thereby regulates the accumulation of H_2O_2 created by polyamine oxidation (Lou *et al.*, 2016). By regulating the positive feedback loop involving H_2O_2 and salicylic acid, NATA1 provides cross-regulation between the salicylic acid defense signaling pathway the jasmonate defense signaling pathway, which induces *NATA1* expression.

A homologous gene, *NATA2* (At2g39020), is located directly adjacent to *NATA1* in the Arabidopsis genome and encodes an enzyme with approximately 80% amino acid sequence identity to NATA1 (Lou *et al.*, 2016). Interestingly, the *NATA1-NATA2* duplication event appears to have taken place only in Arabidopsis and closely related species (Adio *et al.*, 2011; Lou *et al.*, 2016). Other plants, including *Brassica* spp., only have *NATA2* homologs. Moreover, with 25% total sequence identity and highly conserved motifs, NATA2 is the closest Arabidopsis homolog of human spermine/spermidine acetyl transferase (SSAT), which plays a critical role in stress responses (Ignatenko *et al.*, 1996; Tavladoraki *et al.*, 2012). SSAT enzymes with varied substrates and functions also have been identified in bacteria and yeast (Liu *et al.*, 2005; Bai *et al.*, 2011), but not yet in plants.

Although *NATA2* and *NATA1* are likely the result of tandem duplication, they have undergone neofunctionalization, at least at the transcriptional level. Whereas *NATA2* expression is fairly consistent throughout Arabidopsis developmental stages and tissue types, *NATA1* expression is strongest in rosette leaves (http://www.bar.utoronto.ca/; Winter *et al.*, 2007). The metabolic products of NATA1, however, can be found not only in the leaves, but also in the stems, flowers and root tissue (Adio *et al.*, 2011). When it comes to induced responses, *NATA1* expression is up-regulated by a variety of stresses, including cold, salinity, drought, wounding, and jasmonate treatment, but NATA2 is primarily induced by heat stress (http://jsp.weigelworld.org/expviz/; Schmid *et al.*, 2005).

Heat stress is one of the most common abiotic stresses that plants have to contend with. Meanwhile, increased polyamine accumulation has been frequently observed in plants subjected to heat stress (Roy and Ghosh, 1996; Alcazar *et al.*, 2006; Cheng *et al.*, 2009; Sagor *et al.*, 2013;

Shen *et al.*, 2016). In Arabidopsis, Sagor *et al.* (2013) reported that cellular homeostasis of polyamines positively regulates plant heat stress responses and upregulates heat shock proteins (HSPs), as well as heat shock transcription factors, eventually leading to better heat tolerance. This could be due to the stabilizing effect polyamines, especially spermine, have on the mRNA of heat stress responsive genes under high temperature (Shen *et al*. 2016).

Here we provide *in vivo* and *in vitro* evidence that NATA2 is a polyamine acetyltransferase that primarily uses ornithine and spermine as substrates. Upregulation of *NATA2* by heat stress results in fine tuning of the polyamine titer in Arabidopsis cells, which further leads to regulatory effects on Arabidopsis stress responses. We showed that, via NATA2 regulation, Arabidopsis responds to heat stress, as well as pathogen and herbivore attacks under high temperature.

Results

Arabidopsis NATA2 Functions as an Ornithine and Spermine Acetyltransferase

Because of noticeable sequence similarity between NATA2 and NATA1, as well as their striking similarity to mammalian SSAT (Lou *et al.*, 2016), we hypothesized that NATA2 also functions as a polyamine acetyltransferase. To test this hypothesis, we performed *in vitro* assays with Arabidopsis NATA2 purified after transient gene expression in *Nicotiana benthamiana*. Significant free CoA accumulation, indicating successful acetyl group transfer from acetyl-CoA, occurred when ornithine and spermine were provided as substrates for NATA2 (Fig. 3.2). Formation of N^{δ}-acetylornithine and N^{1}-acetylspermine was further confirmed using HPLC-MS. Smaller amounts of free CoA were detected when *N*-acetylputrescine and 1,3-diaminopropane were provided as substrates for NATA2.

The function of the NATA protein was further characterized *in planta* using a *NATA2* knockout line (Salk_092319; *nata2*). The mutant showed no obvious visible defects and initiated flowering at the same time as wildtype Columbia-0 (Col-0) under normal growth conditions. Despite the strong acetyltransferase activity that NATA2 showed in our *in vitro* enzyme assays, we did not observe significant differences in the polyamine and acetylated polyamine profiles between rosette leave extracts of *nata2* and of wild type plants (Fig. 3.3). This might be due to the low expression level of *NATA2* in rosette leaves, especially under non-stressed conditions (http://www.bar.utoronto.ca/; Winter *et al.*, 2007). Moreover, in many cases, we observed low to

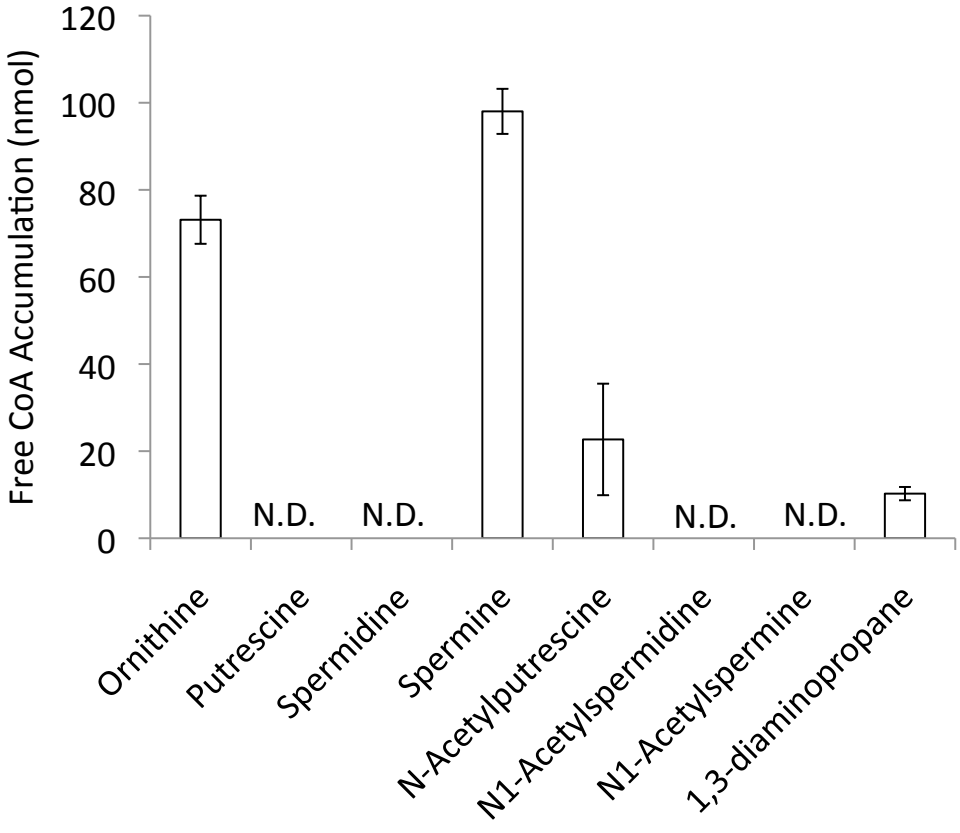

Figure 3.2. NATA2 converts ornithine to N^{δ}-acetylornithine and spermine to N^{1}-acetylspermine. Enzyme activity was measured spectrophotometrically by the release of CoA from acetyl-CoA during the 30 min reaction. Mean +/- s.e. of N = 3.

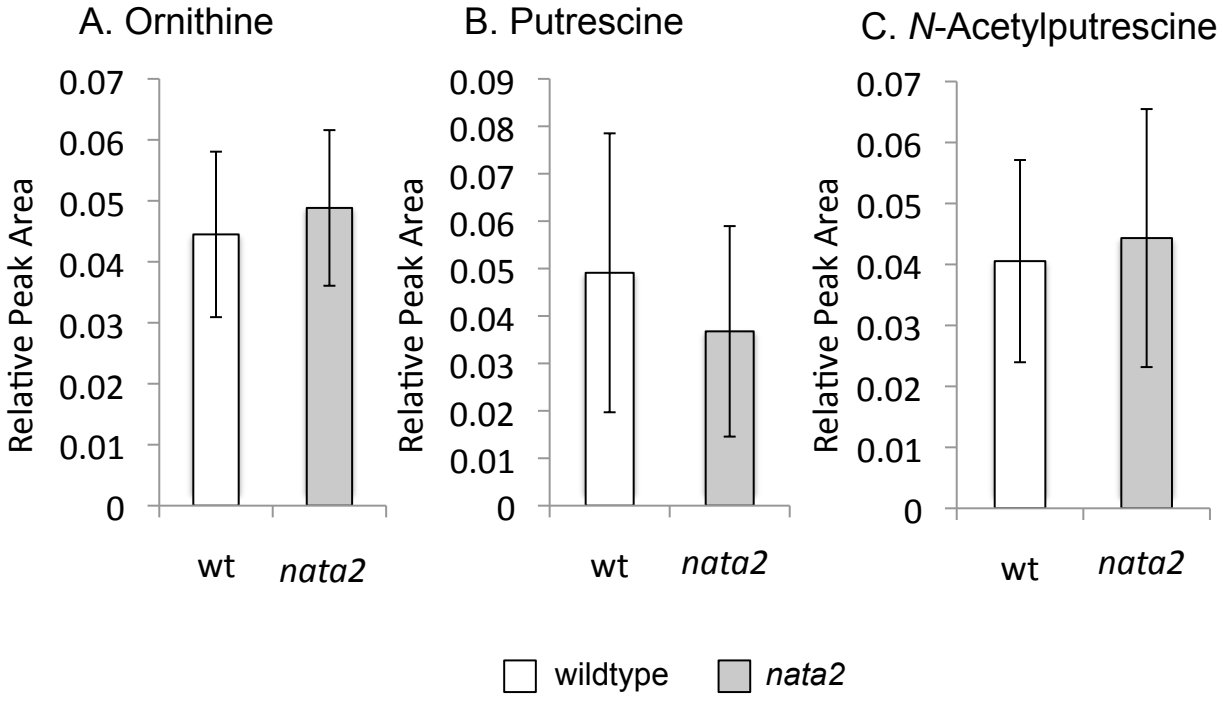

Figure 3.3. Accumulation of polyamines in rosette leaves of wildtype Arabidopsis and *nata2* mutant plants measured by HPLC. (A) ornithine, (B) putrescine, and (C) *N*-acetylputrescine. Mean +/- s.e. of N = 9.

no accumulation of higher polyamines, *i.e.* spermidine and spermine, in the extracts, which might also hinder the observation of the acetylated forms.

NATA2 transcripts are most abundant in imbibed seeds and heat-stressed Arabidopsis (http://www.bar.utoronto.ca/; Winter *et al.*, 2007). Therefore, we performed seed imbibition experiments with wildtype Col-0 Arabidopsis, the *NATA2* knockout line, and a *NATA1* knockout line (GK-256F07; *nata1*). After up to 48 hours imbibition, we observed similar amounts of spermine accumulation in all seeds (Fig. 3.4). However, whereas wildtype and *nata1* seeds sometimes accumulated N^1-acetylspermine, this compound was not detected in *nata2* seeds (Fig. 3.4). In fact, we detected N^1-acetylspermine more frequently in *nata1* samples than in pooled wildtype seeds, suggesting potential redundancy between NATA1 and NATA2.

Polyamine levels, especially spermidine and spermine levels, are known to be tightly regulated in plant tissues. Thus, their acetylated forms are not expected to be abundant. It has been reported that N^1-acetylspermine abundance in Arabidopsis root tissue is comparable to that of spermine (Kamada-Nobusada *et al.*, 2008). To determine whether *NATA2* affects N^1-acetylspermine accumulation, we extracted root tissue from *nata2* and wildtype Arabidopsis for analysis. Although neither spermine nor N^1-acetylspermine were detected under our growth conditions (data not shown), *nata2* roots accumulated a higher level of ornithine, a substrate for NATA2, than roots of wildtype plants (Fig. 3.5). We also observed a higher level of *N*-acetylputrescine, a product of NATA1 activity, while the level of its precursor, putrescine, remained the same in tested plant tissues (Fig. 3.5). Taken together, our *in vitro* and *in vivo* results indicate that NATA2 is an ornithine and spermine acetyltransferase, but also suggest a potential redundancy between the NATA1 and NATA2 enzymatic functions.

The *nata2* Mutant is Less Sensitive to Heat Stress in Hypocotyl Elongation

Despite having strong expression in younger seedlings (http://www.bar.utoronto.ca/; Winter *et al.*, 2007), the potential role of NATA2 in seedling development is largely unknown. In fact, under normal growth conditions, Arabidopsis *nata2* seedlings show no obvious growth phenotype compared to wildtype seedlings (Supplementary Fig S3.1). However, based on the fact that NATA2 is heat-inducible, we hypothesized that NATA2 plays a role in regulating heat stress responses during seed development by fine-tuning polyamine levels.

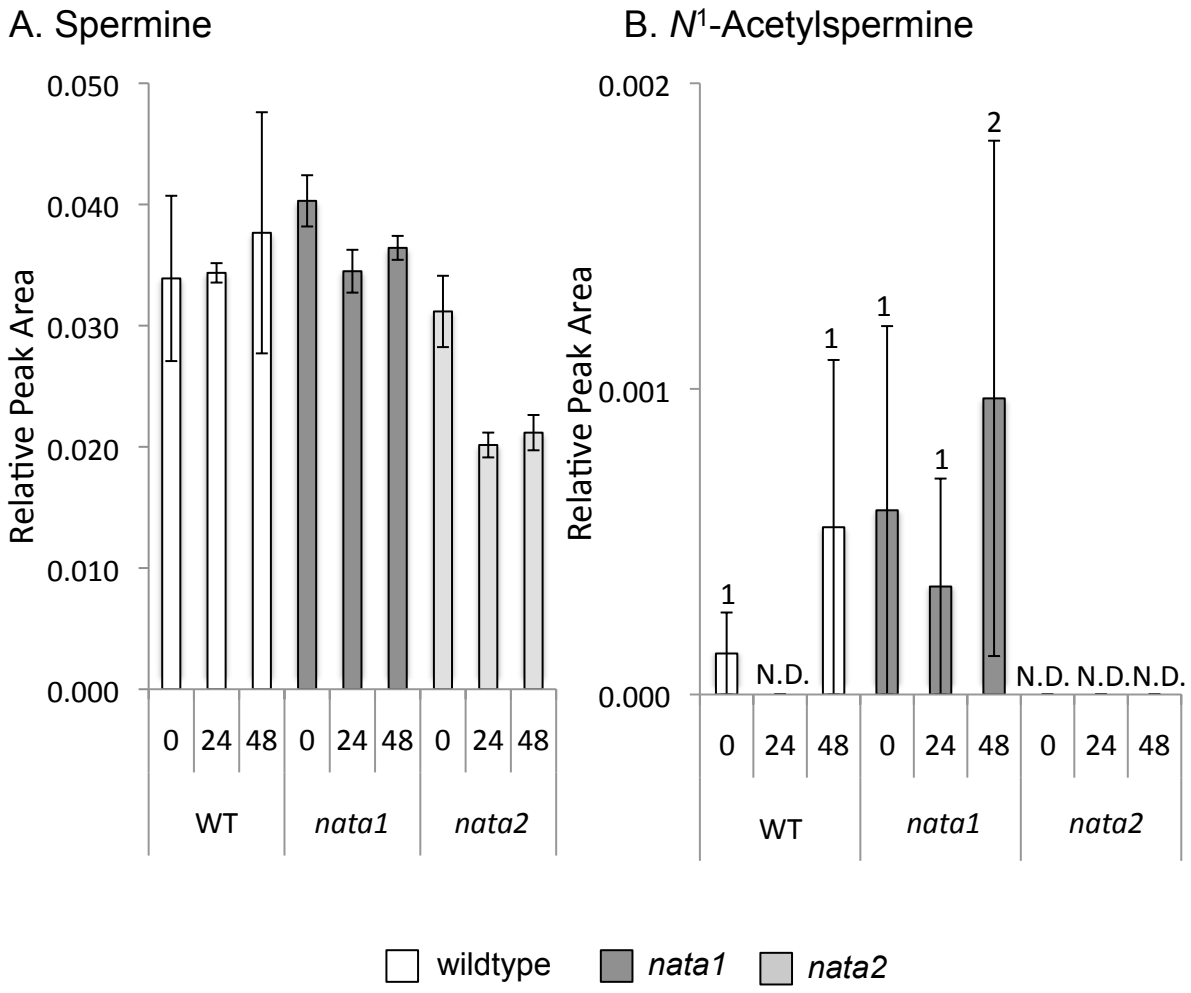

Figure 3.4. (A) Spermine, and (B) N^1-acetylspermine concentrations in seeds of wildtype Col-0 and *nata2* mutant Arabidopsis imbibed for 0 hr, 24 hr and 48 hr in water. Mean +/- s.e. of N = 3. Numbers on the column indicates the number of sample in which N^1-acetylspermine were measured.

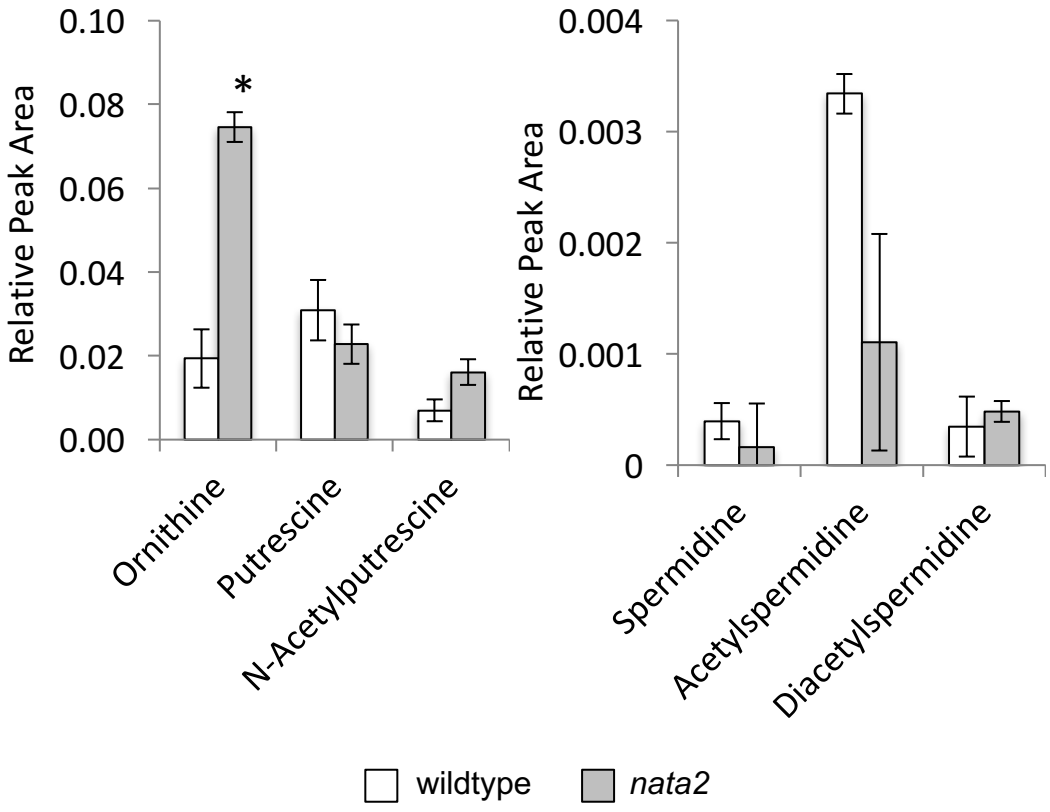

Figure 3.5. Polyamine concentrations in roots of wildtype Col-0 and *nata2* mutant Arabidopsis. Mean +/- s.e. of N = 3.

To test this hypothesis, we performed a hypocotyl elongation experiment similar to one reported by Shen *et al*. (2016). The significantly longer hypocotyl length of *nata2* mutants compared to wildtype Col-0 at 37°C indicates that the mutants are more tolerant of heat stress (Fig 3.6A). No hypocotyl length differences between mutant and wildtype were observed when seedlings were grown at 23°C (Fig 3.6A). To determine whether the observed phenotype is the result of altered polyamine titer due to NATA2 activity, we analyzed the polyamine profile of *nata2* mutant and wild type seedlings after heat stress. As in our previous experiments, accumulation of N^1-acetylspermine was observed infrequently in all tested samples (Fig 3.6B). Somewhat surprisingly, wildtype seedlings accumulated more spermine than *nata2* mutants (Fig 3.6B). While this agrees with our hypothesis that a change of spermine abundance led to differences in the heat stress response between Col-0 wildtype and *nata2* mutant plants, it also suggests a more complicated regulation of polyamine levels in Arabidopsis cells.

To further investigate the *nata2* mutant effects in Arabidopsis seedlings, quantitative RT-PCR assays were used to determine whether known stress-responsive genes are differentially regulated compared to wildtype Col-0. Consistent with previously reported results, *NATA2* expression increased within three hours after the application of heat stress (Fig 3.7A). No *NATA2* transcripts were detected in the *nata2* mutant, confirming the knockout mutation. The expression of *HSP70,* a commonly used marker for the heat stress responses in Arabidopsis, is only marginally higher in the *nata2* mutant than in wildtype (Fig 3.7B). Both groups of seedlings also showed comparable *NATA1* transcript abundance (Fig 3.7C). Interestingly, the *nata2* mutation caused elevated expression of *PR1*, *PR2*, and *PR5,* all of three of which are commonly used markers for pathogen defense responses in Arabidopsis (Fig 3.7D, E, F).

Heat-Mediated Induction of NATA2 Improves *P. syringae* Growth

The observation of increased *PR1*, *PR2*, and *PR5* expression in NATA2-deficient plants indicated elevated defense against biotrophic pathogens. This is in consistent with the fact that polyamine abundance in plant cells is closely related to defense against pathogens (Yoda *et al.*, 2006; Yoda *et al.*, 2009; Kim *et al.*, 2013; Rossi *et al.*, 2015; Lou *et al.*, 2016). Hence, we hypothesized that, given regulatory function of polyamine homeostasis, NATA2 plays a role in crosstalk between abiotic stress and biotic stress response pathways, *i.e.* between heat stress and pathogen defenses.

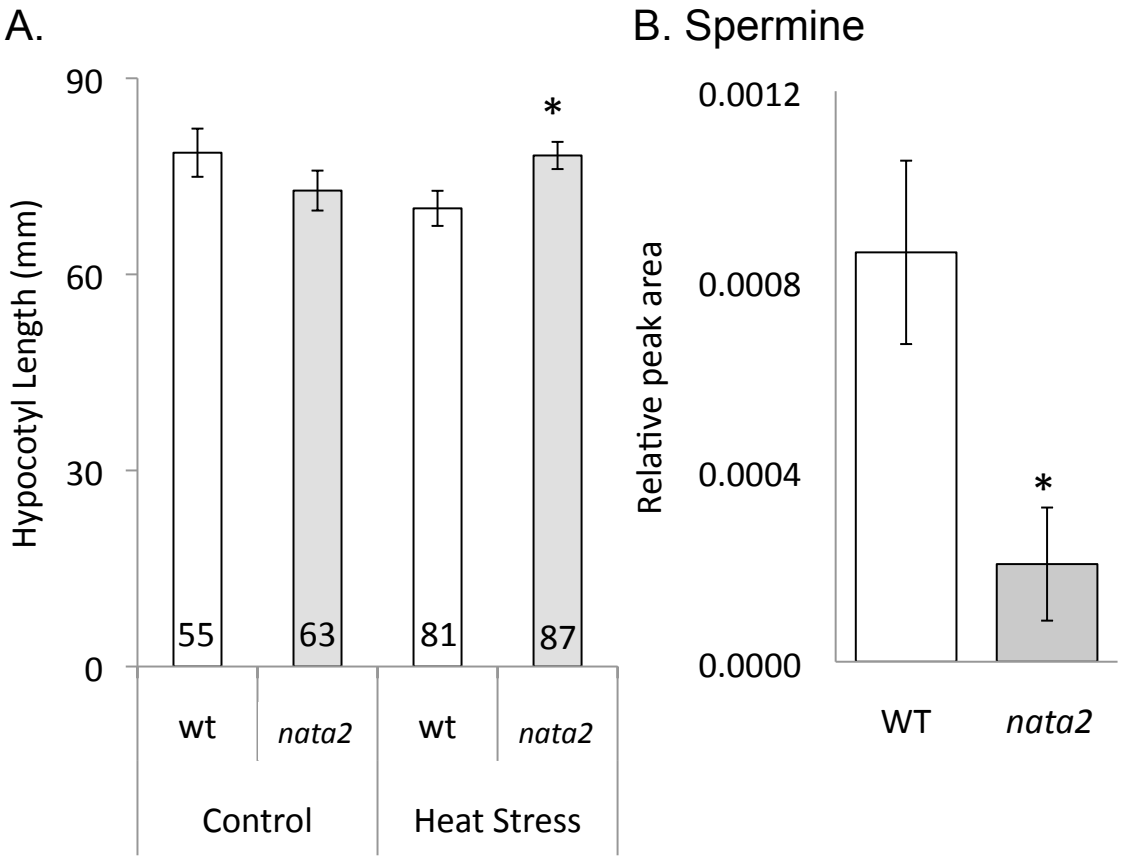

Figure 3.6. (A) Seedlings of *nata2* mutant are less sensitive to heat stress in hypocotyl elongation. Numbers in bars indicate sample sizes ± SE.. *P < 0.05, two tailed *t*-test. (B) Spermine accumulation in 7 days old seedlings 3 days after heat stress. Mean +/-s.e. of N = 4.

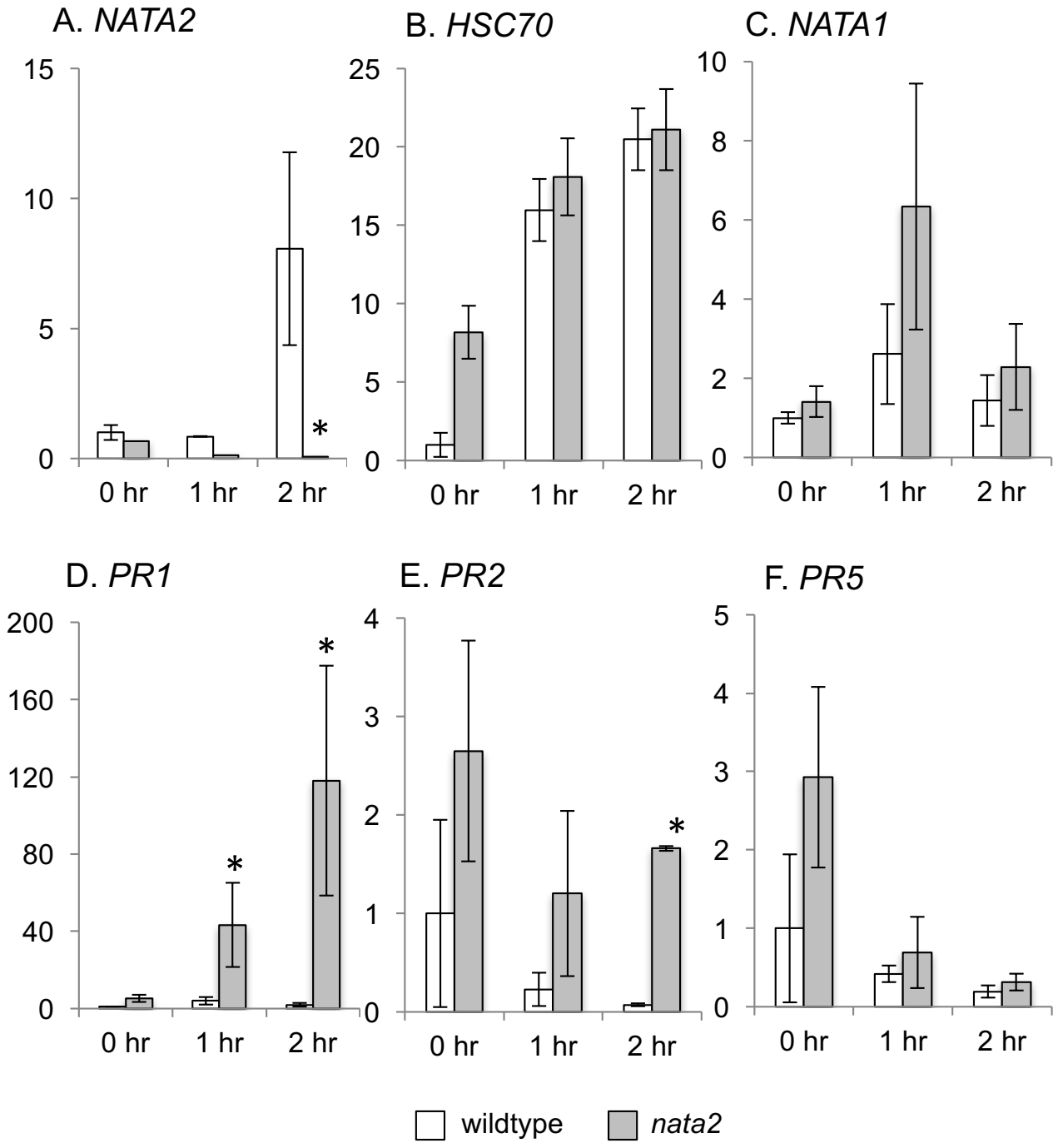

Figure 3.7. Transcript abundance of (A) *NATA2*, (B) *HSP70*, (C) *NATA1*, (D) *PR1*, (E) *PR2*, and (F) *PR5* in wildtype Col-0 and *nata2* mutant seedlings 2 hours after heat stress at 37°C. Gene expression was measured by quantitative RT-PCR. Mean ± SE of two independent experiments, each of N = 3-6. *P < 0.05, two tailed *t*-test comparing expression in wildtype and *nata2* at the same time point.

To test the hypothesis, 18-day-old wildtype and *nata2* mutant Arabidopsis were infected with *P. syringae* at 28°C, a temperature that is unfavorable for normal Arabidopsis growth. Four days after infiltration, bacterial counts were higher in wildtype Col-0 than in *nata2* plants (Fig 3.8A). Moreover, a greater increase in H_2O_2 accumulation was observed in *nata2* mutants compared to wildtype plants (Fig 3.8B). However, we did not observe any differences in plant susceptibility or H_2O_2 accumulation, when the same experiment was performed at 23°3.C (Fig 3.8A, B). Taken together, these results suggest that heat-induced *NATA2* expression, by reducing the amount of H_2O_2 generated by polyamine oxidation, makes plants more susceptible to *P. syringae*.

Heat-Mediated Induction of NATA2 Improves *S. exigua* Growth

It is well known that, within limits, insect herbivores perform better under elevated temperature. However, the effects of temperature on plants that affects herbivore performances remain to be elucidated (Bale *et al.*, 2002). Since polyamines have regulatory functions in many aspects of plant physiology, we hypothesized that a defect in the fine-tuning of polyamine signaling would affect herbivore performance.

Similar to the above pathogen experiment, 18-day-old wildtype and *nata2* mutant Arabidopsis were individually caged with beet armyworms (*Spodoptera exigua*). The *nata2* mutation did not affect caterpillar growth under normal at 23ºC (Fig. 3.9). However, *nata2* mutant plants grown at 28ºC are less suitable for the caterpillars, as is indicated by the reduced weight gain compared to the caterpillars feeding on wildtype plants (Fig. 3.9).

The Potential Redundancy between NATA2 and NATA1

The fact that in the seed imbibition experiments, despite high variability, *nata1* mutant plants accumulated more N^1-acetylspermine than wildtype and *nata2* Arabidopsis indicates a potential redundancy between NATA1 and NATA2. In fact, an LC-TOF-MS metabolite analysis of wildtype, *nata1,* and *nata2* rosette leaves after methyl jasmonate induction also showed significant differences when comparing the induced peak features in wildtype to those in *nata1* and *nata2* (Fig 3.10). These results indicate that NATA1 and NATA2 partially compensate the loss of each other, or at least act on the same or closely related pathway(s). Hence, it is desirable to have a double knockout mutant for further investigation. As both genes are directly adjacent to on an-

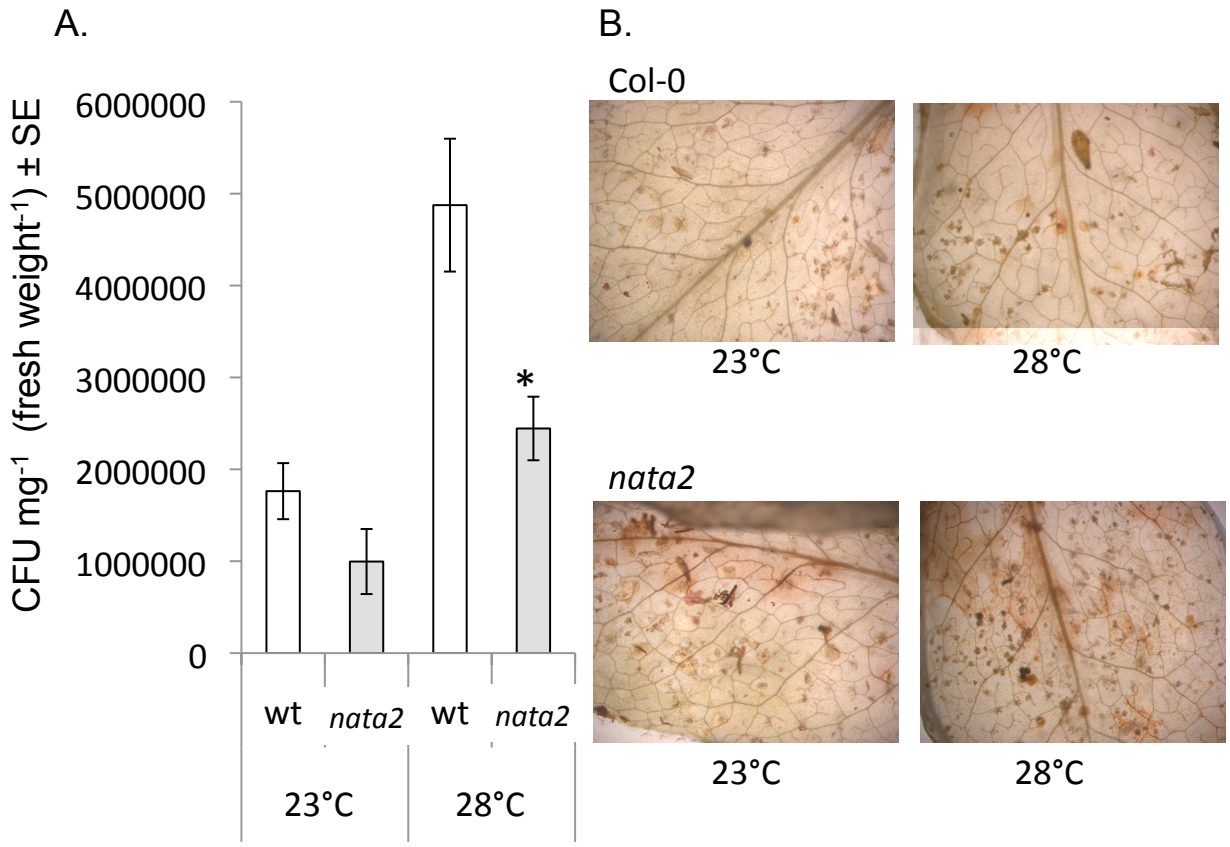

Figure 3.8. Effects of *NATA2* on *P. syringae* growth under 28°C heat stress. (A) Bacterial titers in wildtype Col-0 and *nata2* rosette leaves after infiltrating 10^5 CFU ml^{-1} culture of DC3000. Mean ± SE, N = 16. *P < 0.05, ANOVA followed by Tukey's HSD test. (B) Representative hydrogen peroxide staining of infected leave tissue after four days.

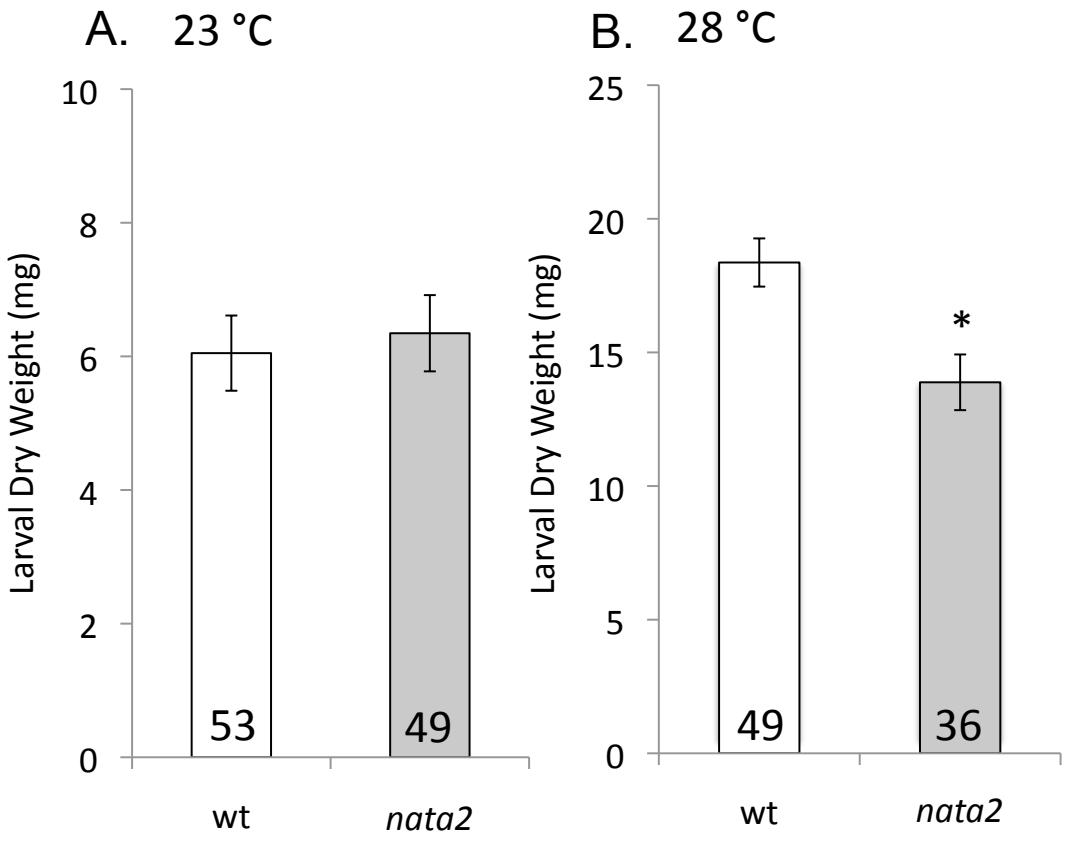

Figure 3.9. Heat Stress Induced *NATA2* Improves *S. exigua* growth. Larval weight gain at (A) 23°C (B) 28°C. Numbers in bars indicate sample sizes ± SE.. *P < 0.05, two tailed *t*-test.

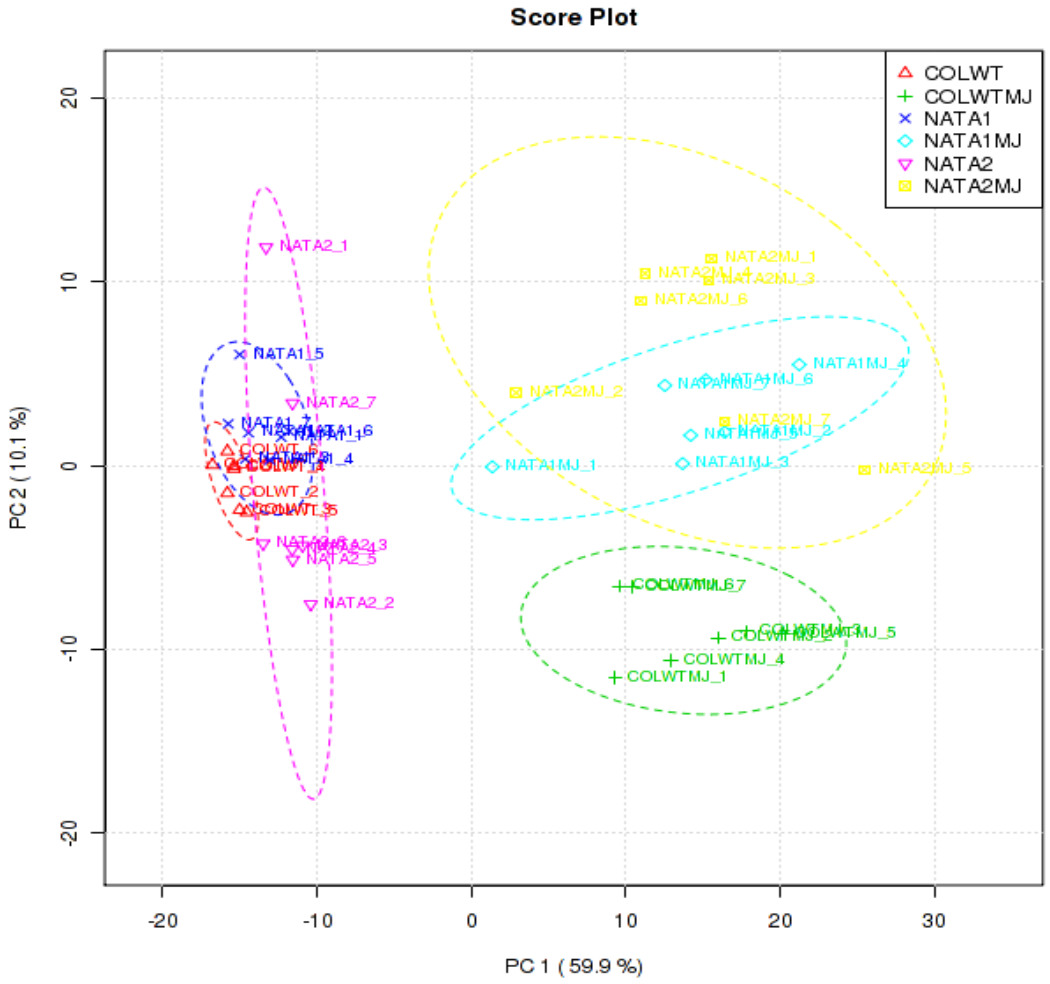

Figure 3.10. Principal component analysis of HPLC-TOF-MS metabolite profiling data of wildtype Col-0, *nata1* and *nata2* rosette leaves, control and four days after MeJA treatment. Seven measurements of each treatment condition are shown.

other, the infrequency of meiotic recombination limited the use of traditional crossing techniques. Therefore, we made use of Transcription Activator-Like Effector Nucleases (TALENs), an effective method for site-directed mutagenesis (Cermak *et al.*, 2011), to create a *NATA2* knockout mutation in a *nata1* mutant background (Christian *et al.*, 2013).

However, after multiple generations of screening we did not obtain any homozygous *nata1 nata2* double mutants. In fact, in both lines that have confirmed a loss of the 35S:TALEN insertion, we observed a reduced germination rate (69% and 77%, respectively). This is not significantly different from the 3:1 live:dead ratio that is expected from this cross if the *nata1 nata2* double mutation is lethal early in development ($P < 0.05$, chi-squared test). The germinated plants show no obvious phenotype comparing to wildtype Arabidopsis, as is the case for *nata1* and *nata2* single mutants. Taken together, these indicate that NATA1 and NATA2 have essential functions in Arabidopsis seedling germination and the loss of both of genes is lethal.

Discussion

While the functions of acetylated polyamines have been extensively studied in bacteria and mammals, their function in plants is less clear. Our results show that NATA2, an ornithine and spermine acetyltransferase, alters plant defenses by regulating free polyamine levels. When *NATA2* expression is induced by heat stress, this regulation interferes with plant defenses against pathogens and herbivores. In particular, *NATA2* upregulation counteracts the effects of spermine accumulation under various stresses (Sagor *et al.*, 2013; Mostofa *et al.*, 2014; Shen *et al.*, 2016). Although we failed to observe an obvious benefit for plants to having *NATA2*, we feel confident in the assumption that NATA2 assists in maintaining cell polyamine and H_2O_2 homeostasis, as is the case for NATA1. In fact, it has been shown that NATA1, by acetylating polyamines, regulates polyamines abundance without generating an H_2O_2 burst, as is the case for polyamine degradation by polyamine oxidases (Lou *et al.*, 2016). Our current research shows a similar regulation of polyamine and H_2O_2 accumulation by NATA2 activity in Arabidopsis.

Cellular polyamine homeostasis is tightly controlled, not only by polyamine biosynthesis, but also by its transport, sequestration, and H_2O_2-generating catabolism (Moschou *et al.*, 2008; Tiburcio *et al.*, 2014; Shen *et al.*, 2016). Polyamine catabolism is mediated by two classes of polyamine oxidases, the copper amine oxidases (CuAOs; EC 1.4.3.6) and the flavin-containing polyamine oxidases (PAOs; EC 1.5.3.11) (Bagni and Tassoni, 2001; Moschou *et al.*, 2008;

Moschou *et al.*, 2008; Tavladoraki *et al.*, 2012). In Arabidopsis, ten genes encode putative CuAOs (Planas-Portell *et al.*, 2013; Tavladoraki *et al.*, 2016) and five encode PAOs (PAO1 - PAO5)(Fincato *et al.*, 2011; Kim *et al.*, 2014). Whereas the activity of individual CuAOs remains to be characterized (Planas-Portell *et al.*, 2013), it is well-documented that the five PAO proteins show differences in their activities toward specific polyamines (Fincato *et al.*, 2011; Fincato *et al.*, 2012; Kim *et al.*, 2014). For example, the activity of PAO1 toward N^1-acetylspermine is significantly lower than toward spermine, thermospermine and norspermine (Fincato *et al.*, 2011). On the other hand, PAO5 has been documented to preferentially act on N^1-acetylspermine over spermine and spermidine (Ahou *et al.*, 2014; Kim *et al.*, 2014), whereas N^1-acetylspermine likely functions as a negative effector on PAO5 at the same time (Kim *et al.*, 2014). All the other PAOs tested, even PAO2, which showed the highest activity with N^1-acetylspermine, still preferentially utilize non-acetylated free spermine by a five-fold ratio (Fincato *et al.*, 2011). This is consistent with our observation that, with increased activity of NATA2, which competes with spermine degradation, there is less H_2O_2 accumulated after pathogen infection. However, the fact that little or no N^1-acetylspermine accumulated throughout our experiments suggests that these compounds are being rapidly degraded or conjugated. Hence, to what extent the acetylation is changing the production of H_2O_2 might be largely affected by the spatial and temporal expression of the specific PAO. In addition, further investigation of the CuAO substrate preferences, as well as expanding the tested PAO substrates to various acetylated forms, for example N^1N^{12}-diacetylspermine, would be beneficial for understanding the catabolic pathway for acetylated polyamines.

Despite much being known about the function of SSAT-mediated polyamine acetylation, how polyamine acetylation, directly or indirectly, regulates stress responses in plants requires further investigation. Mammalian SSAT catalyzes the formation of N^1-acetylspermidine, N^1-acetylspermine and N^1N^{12}-diacetylspermine (Ignatenko *et al.*, 1996; Tavladoraki *et al.*, 2012), and by doing so allows these acetylated polyamines, which have reduced positive charges, to pass through membranes, resulting in regulatory effects on many aspects of animal metabolism (Tavladoraki *et al.*, 2012). Arabidopsis NATA2, on the other hand, only showed *in vitro* enzyme activity with ornithine and spermine as substrates. We also did not detect N^1N^{12}-diacetylspermine in Arabidopsis, although this could be an effect of the generally low spermine level of observed acetylation. The differences in substrate preferences is also in agreement with the amino acid se-

quence comparisons between SSAT, NATA1 and NATA2 (Lou *et al.*, 2016). Although the catalytic site and acetyl-CoA binding pocket are conserved within all three enzymes, there is more variation in the substrate binding site, perhaps explaining the observed shift in the acetylation substrates. Nevertheless, to the best of our knowledge, it remains to be investigated whether acetylation promotes polyamine transport in plants, as it does in mammals.

The challenge we came across in detecting striking polyamine profile differences in *NATA2*-deficient Arabidopsis could be the result of partial complementation by the duplicated homologue, *NATA1*. Although both *nata1* and *nata2* null mutants are viable (Fig. 3.7A; Adio *et al.* 2011), we did not obtain a *nata1 nata2* double knockout mutant, instead observing reduced germination and segregation consistent with lethality of the double mutant in both tested lines. This indicates that NATA1 and NATA2 are essential for plant survival. However, since NATA1 and NATA2 show differences both in substrate preferences and gene transcriptional regulation, how, or how well, one can compensate the loss of another remains to be understood.

The *NATA1-NATA2* duplication is present only in a small number of plant species, including Arabidopsis. However, it is common to find two or more *NATA* gene copies in the Brassicaceae (Adio *et al.*, 2011; Lou *et al.*, 2016). While the *NATA* gene duplication has been related to the loss of ornithine decarboxylase in the *Brassicaceae*, plants with functional ornithine decarboxylase, including *Solanum lycopersicum*, *Solanum tuberosum* and *Populus trichocarpa*, also have more than one *NATA* homologue (Lou *et al.*, 2016), suggesting a benefit from having multiple polyamine acetyltransferases. Further investigation of different plant systems would be useful for understanding the ubiquitous function of polyamine acetylation.

Interestingly, *NATA2* upregulation inhibits seedling elongation under heat stress. At the same time, the *nata2* mutants, being more heat resistant, accumulated less spermine than wildtype seedlings. This is somewhat surprising, as prior studies showed that *in planta* accumulation and external treatment of spermine helps plants to tolerate heat stress (Sagor *et al.*, 2013; Shen *et al.*, 2016). However, we cannot rule out that N^1-acetylspermine plays a similar heat stress rescuing function, nor is it known whether N^1-acetylspermine is a precursor for the production other plant metabolites that promote heat resistance. In fact, our results suggest that regulation of the polyamine pathway in response to stress is more complicated than previously suspected. For example, steady-state polyamine accumulation does not provide information about the metabolic flux through this pathway. Also, conjugated polyamines and other polyamine-derived

compounds need to be taken into consideration to provide a more complete picture of polyamine metabolic networks.

Conjugated polyamines are particularly abundant in plant reproductive tissue, which tends to be well-defended. In fact, the high titer of polyamine conjugates in seeds is believed to serve as a nitrogen sink for use during germination (Moschou *et al.*, 2012). Defense-induced production of conjugated polyamines also has been reported in several plants and has a direct role in defense against lepidopteran herbivory (Kaur *et al.*, 2010). Our results show that, by changing polyamine levels in plants, polyamine acetylation also plays a role in defense against herbivory during heat stress. The fact that NATA2 is heat-induced, but can infer with herbivory resistance and plant resistance against pathogens, again emphasis that polyamine signaling is critical for shifting plant responses to multiple biotic and abiotic stresses.

Taken together, we have provided evidence that, by inducing polyamine acetylation, plants have the ability to control cellular homeostasis of free polyamines without undesirable H_2O_2 formation. By doing so, plants can modulate responses to multiple stresses. This suggests a critical role for polyamines in plant defense signaling. Further studies that include experiments with conjugated polyamines would greatly improve our understanding on this plant defense signaling pathway.

Methods

Plants and growth conditions

Seeds of wildtype Columbia-0 Arabidopsis, *nata2* (Salk_092319, Rosso *et al*., 2003), and *nata1* (GK-256F07, Alonso *et al.*, 2003) were obtained from the Arabidopsis Biological Resource Center (www.arabidopsis.org). Plants were grown in Cornell mix (by weight 56% peat moss, 35% vermiculite, 4% lime, 4% Osmocoat slow-release fertilizer [Scotts, Marysville, OH], and 1% Unimix [Scotts]) in 20x40-cm nursery flats in Conviron growth chambers with a photosynthetic photon flux density of 200 mmol m-2 s-1 and a 16:8 h day:night photoperiod, at 23°C with a 50% relative humidity. For experiments carried out at elevated temperature, a chamber set to identical conditions except for at 28°C was used.

Protein extraction and *in vitro* assays

For protein purification, NATA2 was cloned behind the cauliflower mosaic virus 35S promoter in the T-DNA binary vector pYL436, as described by Rubio *et al.*, 2005, and transformed into *Agrobacterium tumefaciens* strain GV3101 for *N. benthamiana* transient expression. Primers used for cloning are listed in Supplementary Table S3.1. A pYL436 empty vector was included in the experiments as a control. Proteins were further purified by immunoprecipitation as described in Brauer *et al.*, 2014, with slight modification. In short, fully expanded *N. benthamiana* leaves were infiltrated with a mixture of *A. tumefaciens* strains carrying the desired construct and turnip crinkle virus capsid protein (P38) using a 1 ml needle-less syringe. Leaf tissue was collected three days after infiltration and ground to fine powder in liquid nitrogen. All following purification steps were carried out in a 4°C cold room. After mixing with extraction buffer (100 mM Tris-HCL, pH 7.5, 100 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.1% Triton-X, 1% β-mercaptoethanol, 10% glycerol and 1mM PMSF), cell debris was removed by centrifugation (3,600 x g for 10 min). The supernatants were mixed with IgG Sepharose® 6 Fast Flow (GE Healthcare Lifescience; http://www.gelifesciences.com/) for 2-4 hours. Beads were then washed with the same buffer containing 500 mM NaCl before being incubated with Turbo3C™ Protease (Accelagen; http://www.accelagen.com/) in cleavage buffer (50 mM Tris-HCL, pH 7.0, 150 mM NaCl, 1 mm EDTA, 1 mM DTT, 1% Triton-X) overnight. Supernatants were harvested the next morning and incubated with Glutathione-Superflow Resin (Clontech; http://www.clontech.com/) for 1 hour with gentle rotation. Protein purified using this method was aliquoted and stored as 25% glycerol samples at -80°C. Prior to *in vitro* experiments, total protein content was quantified by Bradford assay, and a single protein band could be observed by SDS-polyacrylamide gel electrophoresis with silver staining.

Enzymatic activity toward potential substrates was determined as described in Lou *et al.*, 2016. In brief, 2 μg of purified protein were incubated with potential substrates and acetyl-CoA as the acetyl-group donor, in 100 mM Tris-HCl (pH 7.5), 2 mM EDTA (pH 7.5) and 10% glycerol at 30°C for 30 min. Two mM 5-5'-dithiobis[2-nitrobenzoic acid] was added at the end of each reaction to allow spectrophotometric measurement of the free CoA that was generated. In addition to reactions without substrates or acetyl-CoA, an empty vector was used as a negative control. Samples were further derivitazed and analyzed by HPLC, as described below, for confirmation of the expected product.

Methyl jasmonate induction

Methyl jasmonate induction is performed as described previously (Lou *et al.*, 2016), with slight modification. In short, the leaves of 4-week-old Arabidopsis plants were sprayed with an aqueous solution containing 0.01% (v/v) Tween 20, and 0.01 mM methyl jasmonate. Control plants were treated with water containing 0.01% Tween 20 and 0.03% acetone, the solvent used for methyl jasmonate. Plants were covered with plastic domes and tissue were harvested 4 days after elicitation and immediately frozen in liquid nitrogen.

Quantification of polyamines

Polyamines were extracted and derivatized with 6-aminoquinolyl-N-hydroxysuccinimidylcarbamate (AQC) using the AccQ-Fluor reagent kit (Waters) prior to HPLC detection, as described in Lou *et al.*, 2016. For better sensitivity and further confirmation on expected compounds, a 4-(N,N-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F) derivatization method modified from Tsutsui *et al.*, (2013) was used prior to HPLC-MS detection. In short, plant tissue was pulverized in liquid nitrogen and extracted with 25 mM HCl (5 ml mg^{-1} of tissue) containing 25 µM 1,6-hexanediamine as an internal standard. After centrifuging at 15,000 x g at 10°C for 20 min, the supernatants (12 µL each) were mixed with 24 µL of 0.2 M sodium tetraborate. Equal volumes of DBD-F solution, dissolved in acetonitrile, were added to each sample prior to 30 min incubation at 60°C. The reaction mixtures were filtered and analyzed on a Waters® ACQUITY UPLC® BEH C18 column (Waters) using a Thermo Q-Exactive Orbitrap coupled to Dionex ultra-high-pressure chromatography systems for reverse-phase separation with column temperature set at 40 °C. The start condition were 80% solvent A (0.1% formic acid in water) and 20% solvent B (acetonitrile) with flow rate at 0.5 mL/min. The elution gradient was as follows: ramp to 90% solvent B at 5.5 min with curve set to 5 and hold at 90% solvent B till 7 min. The detection conditions were as follows: Heated electrospray ionization mode; 4000 V spray voltage; 320 °C capillary temperature; 70 units sheath gas flow rate; mass range of m/z 80 - 1200 Da. Analytical software (Therm Xcalibur v3.0) was used for the system control and data processing.

Hypocotyl elongation assay

To test the sensitivity of hypocotyl elongation to heat stress, an experiment similar to that described by (Shen *et al*., 2016) was performed. In short, seeds were germinated on half-strength Murashige and Skoog medium (½MS salts, 1.5% sucrose, pH 5.7) with 1.2% agar and left to grow in a vertical position in a dark chamber (23°C). After three days, the plates were sealed and immersed in a 37°C water bath for three hours before transferring back to the dark chamber for another three days of growth in a vertical position. Photographs were taken with a Nikon D80 digital camera and the length of the hypocotyls were measured with ImageJ (imagej.net/).

Quantitative PCR

Relative transcript abundances of *NATA2* (At2g39020), *HSP70* (*NATA1* (At2G39030), *PR1* (At2g14610), *PR2* (At3g57260) and *PR5* (At1G75040) were analyzed and compared by qRT-PCR using actin (At3g18780) as an endogenous control as described in Chapter 2. Gene-specific primers were designed using Primer-Blast (www.ncbi.nlm.nih.gov/tools/primer-blast/) and listed in Supplementary Table S2. Rosette leaf samples were ground in liquid nitrogen and total RNA was extracted using the SV total RNA isolation system (Promega, www.promega.com). After quantification with a Nanodrop system (www.nanodrop.com), 1 µg of total RNA was reverse transcribed using SMART® MMLV Reverse Transcriptase (Clontech, www.clontech.com) using oligo$(dT)_{15}$ primers. Following cDNA synthesis, the samples were diluted in nuclease free water and used for qRT-PCR reactions with the SYBR® Green PCR master mix (Applied Biosystems, www.appliedbiosystems.com) using an Applied Biosystems 7900HT Instrument. Each reaction was carried out with the following conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 15 s and 72°C for 15 s, and final extension at 72°C for 2 min. The C_T values were quantified and analyzed according to the standard curve method.

Bacterial growth assays

Pseudomonas syringae strain DC3000 was obtained from N. Clay (Yale University, New Haven, CT), and an infection experiment was performed as described by Lou *et al*. (2016). In short, bacteria were cultured at 30°C in Luria-Bertani (LB) medium supplemented with 50 µg ml^{-1} rifampicin and 50 µg ml^{-1} kanamycin. Overnight cultures were centrifuged at 3000 x g for

10 min, resuspended, and diluted in water to approximately 10^5 colony forming units (CFU) ml^{-1} before infiltration into three-week-old Arabidopsis rosette leaves with a needle-less syringe. Eight-mm diameter leaf discs were collected 2 days after infiltration and submerged in 1 ml sterile water with 0.01% Tween 20 for 4 h to allow equilibration of the bacteria between the apoplastic space of the leaf disks and the surrounding water, as described previously (Adio *et al.*, 2011). Serial dilutions of the suspensions were spotted on LB agar plates supplemented with 50 μg ml^{-1} rifampicin, and bacterial colonies were counted after 2 days of incubation at 30°C.

H_2O_2 Detection and quantification

H_2O_2 was detected and quantified by 3.3'-diaminobenzidine (DAB) staining as described previously (Daudi and O'Brian, 2012).

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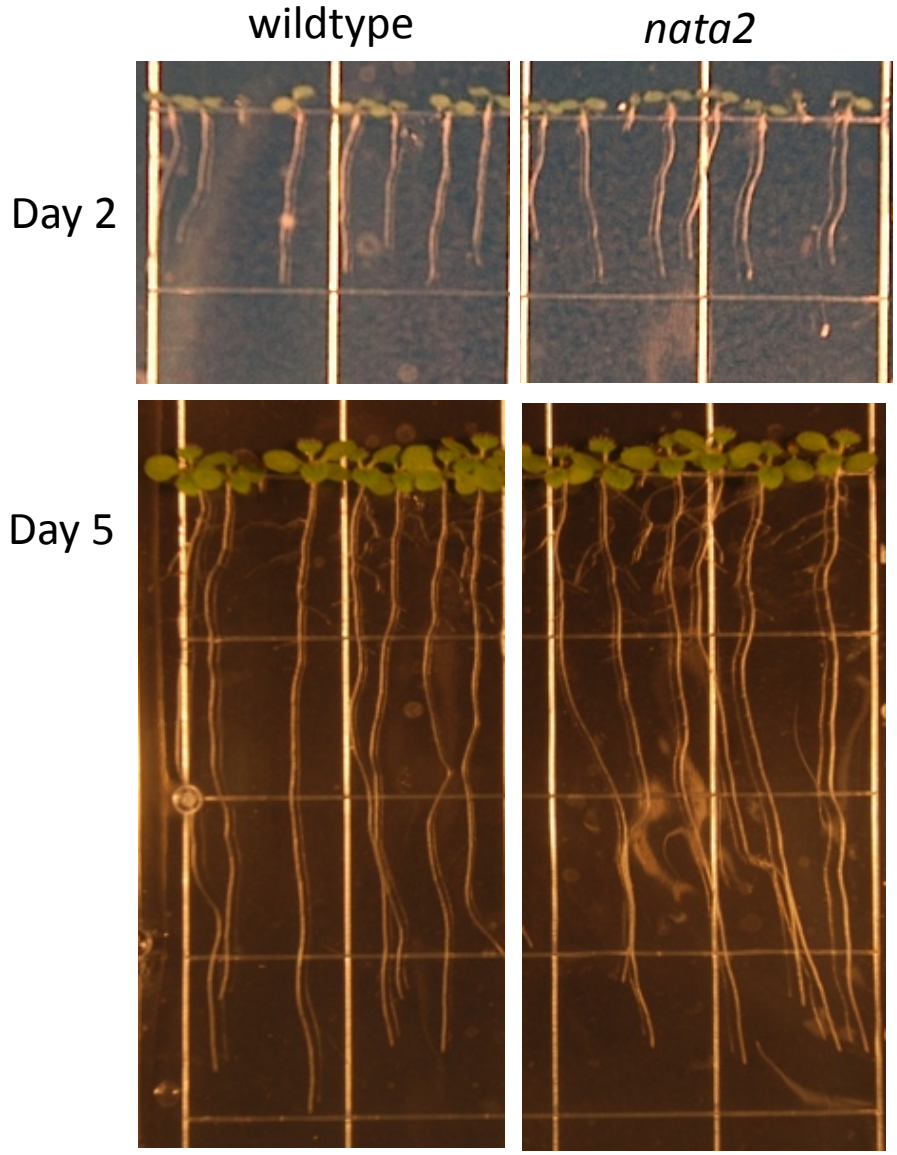

Supplementary Figure S3.1. Arabidopsis *nata2* seedlings show no obvious growth phenotype compared to wildtype seedlings after 2 and 5 days growth on half-MS agar plates.

Supplementary Table S3.1. Primers used for cloning and genotyping.

Target Gene	Purpose		Primer sequence
NATA2 (At2g39020)	Cloning	forward	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CAT GGC AGC CGC CGC ACC G
	Cloning	reverse	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC CTA GAT GTT GAC CTG ATC AAA AGC TTC
SALK-LBa1	Confirm T-DNA insertion	SALK primer	TGGTTCACGTAGTGGGCCATC G
NATA2 (At2g39020)	Confirm T-DNA insertion	T-DNA forward	TCA ACT CAC ACA CAT CGT GTG
NATA2 (At2g39020)	Confirm T-DNA insertion	T-DNA reverse	AAC AAC CCA TTC CAC TCT TCC
NATA2-forward	Confirm NHEJ mutagenesis	forward	TGG GTT TGT TCT GTT TTT C
NATA2-reverse	Confirm NHEJ mutagenesis	reverse	TCTCAGCAACACAGCATCAA
Hyg resistant gene	Confirm TALEN	forward	GCT CCA TAC AAG CCA ACC AC
	Confirm TALEN	reverse	CGA AAA GTT CGA CAG CGT CTC

Supplementary Table S3.2. Primers used for quantitative RT-PCR.

Target Gene		Primer sequence
HSP70.1 (At5g02500)	forward	GGGGAAGATTTTGACAACA
	reverse	TCTTCGCTCTCTCACAGGAAG
PR1 (At2g14610)	forward	TGATCCTCGTGGGAATTATGT
	reverse	TGCATGATCACATCATTACTTCAT
PR2 (At3g57260)	forward	AGCTTCCTTCTTCAACCACACAGC
	reverse	TGGCAAGGTATCGCCTAGCATC
PR5 (At1G75040)	forward	AGCAATGCCGCTTGTGATGAAC
	reverse	ATCACCCACAGCACAGAGACAC
NATA2 (At2g39020)	forward	CGATTGATGATCCGGAGAGT
	reverse	AGCGGTGAAGATGGGTTATG
NATA1 (AT2G39030)	forward	AGCAGATGGGTGCGCAGGTT
	reverse	TCGCTCGATGGGTCTCATGCA
ACT2 (At3g18780)	forward	TCCCTCAGCACATTCCAGCAGAT
	reverse	AACGATTCCTGGACCTGCCTCATC

CHAPTER FOUR

A PATHWAY FOR POLYAMINE BIOSYNTHESIS FROM N^{δ}-ACETYLORNITHINE IN *ARABIDOPSIS THALIANA*[*]

Abstract

Polyamines are small aliphatic amines found in almost all known organisms, ranging from bacteria to plants and animals. In most plants, putrescine, the metabolic precursor for longer polyamines, such as spermidine and spermine, can be produced from arginine, with agmatine or ornithine as intermediates. Ornithine decarboxylase, which converts ornithine to putrescine, is absent in *Arabidopsis thaliana* (Arabidopsis). Here we show that ADC1, one of two known arginine decarboxylases in Arabidopsis, primarily functions as an N^{δ}-acetylornithine decarboxylase. Hence, ADC1 not only synthesizes agmatine from arginine, but also converts N^{δ}-acetylornithine to *N*-acetylputrescine, thereby providing Arabidopsis with another pathway for polyamine biosynthesis. Phylogenetic analysis indicates that duplication and neofunctionalization of *ADC1* and *NATA1*, the enzyme that synthesizes N^{δ}-acetylornithine in Arabidopsis, co-occur in a small number of plants in Brassicaceae, a plant family that is known to have lost the ornithine decarboxylase pathway for polyamine biosynthesis. Unlike ADC2, which is localized in the chloroplasts, ADC1 is in the endoplasmic reticulum together with NATA1, an indication that these two enzymes share a common substrate pool. Furthermore, we show that, upon *Pseudomonas syringae* infection, the genes in the agmatine-dependent polyamine biosynthesis pathway are highly upregulated in *nata1*, *nata1 adc1*, and *nata1 adc2* mutants, whereas those in wildtype plants are only slightly induced. Together, these results are consistent with the model whereby NATA1 and ADC1 together provide a pathway for polyamine biosynthesis in Arabidopsis.

[*]Yann-Ru Lou designed and performed most of the experiments with Ms. Hannah Powell's assistance. Localization experiment was carried out by Professor Paul Morris and his lab member Ms. Sheaza Ahmed. Dr. Jian Yan generated the double mutants, Dr. Adio Wale synthesized the isotope labeled compounds and Dr. Navid Mohaved assisted with mass spectrometry analysis. This article was written with help from Dr. Georg Jander.

Introduction

Polyamines, cationic metabolites that are found in almost all living organisms, play a central role in numerous aspects of plant physiology. Plant growth, development, and many abiotic and biotic stress responses require polyamine signaling (Evans and Malmberg, 1989; Galston *et al*., 1997; Kusano *et al*., 2007; Kusano *et al*., 2008; Takahashi and Kakehi, 2009; Wimalasekera *et al*., 2011; Sagor *et al*., 2015). Changes in plant polyamine content influence transcription, RNA modification, protein synthesis, enzyme activity, and signaling pathways involving jasmonic acid, salicylic acid, abscisic acid, gibberellin, and nitric oxide. However, despite the wide-ranging importance of polyamines in plant metabolism, numerous aspects of polyamine synthesis and catabolism remain under-investigated.

In most plants, putrescine is synthesized via two metabolic pathways (Fig. 4.1A): from ornithine by ornithine decarboxylase (ODC; EC 4.1.1.17), or from arginine by the sequential action of arginine decarboxylase (ADC; EC 4.1.1.19), agmatine iminohydrolase (AIH; EC 3.5.3.12) and carbamoylputrescine amidase (CPA; EC 3.5.1.53) (Bagni and Tassoni, 2001; Alcazar *et al.*, 2010; Tiburcio *et al.*, 2014). Similar to what is observed in many microorganisms, synthesis of putrescine from arginine in plants also may be catalyzed by a combination of ADC and agmatine amidohydrolase (EC 3.5.3.12) (Ariyaratne, 2014). Spermidine is synthesized from putrescine by spermidine synthase (SPDS; EC 2.5.1.16) through the addition of an aminopropyl group from *S*-adenosyl-3-(methylthio)propylamine (decarboxylated *S*-adenosylmethionine), which is produced by *S*-adenosylmethionine decarboxylase (EC 4.1.1.50) (Bagni and Tassoni, 2001; Alcazar *et al.*, 2010; Tiburcio *et al.*, 2014). To form spermine and thermospermine, another aminopropyl group from the same source is added by spermine synthase (EC 2.5.1.22) or thermospermine synthase (EC 2.5.1.79), respectively (Knott *et al*., 2007).

Through a combination of genome comparisons and mutant screens, most enzymes of the basal polyamine biosynthesis pathway have been identified in *Arabidopsis thaliana* (Arabidopsis). However, no Arabidopsis homolog of known *ODC* genes from other plants has been found. An absence of ODC enzymatic activity in Arabidopsis also has been reported (Hanfrey *et al*., 2001). Thus, unlike most plants, Arabidopsis was predicted to make polyamines only via the agmatine branch of the pathway shown in Figure 4.1. Loss of ODC activity has been reported in only a few plant species, including other Brassicaceae and the moss *Physcomitrella*

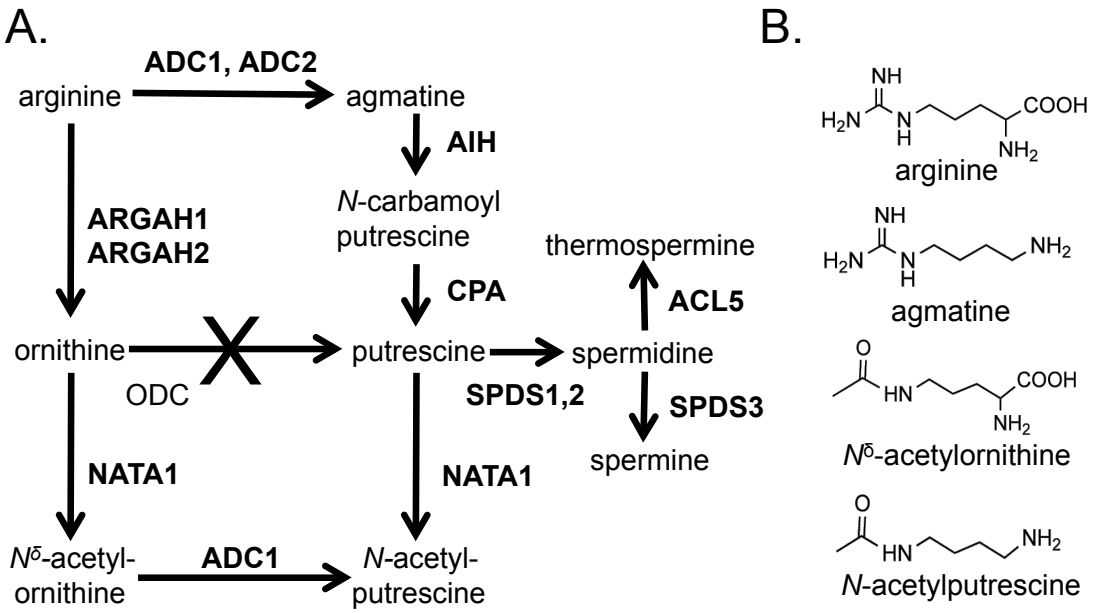

Figure 4.1. (A) Pathways for polyamine synthesis and catabolism in plants. Names of known Arabidopsis proteins catalyzing the reactions are indicated. No gene encoding ornithine decarboxylase has been found in Arabidopsis (B) Structures of arginine, agmatine, and N^{δ}-acetylornithine, and *N*-acetylputrescine.

patens (Fuell *et al*., 2010).

Two Arabidopsis genes, *ADC1* (At2G16500) and *ADC2* (At4G34710) encode ADC activity, catalyzing the formation of agmatine as the first and rate-limiting step in polyamine biosynthesis from arginine (Kumar *et al*., 1997; Watson *et al*., 1998; Hanfrey *et al*., 2001). Although *ADC1* and *ADC2* encode proteins with approximately 80% amino acid sequence identity, their expression patterns vary under different environmental conditions. *ADC1* expression is more limited, in terms of both Arabidopsis development and tissue type, than *ADC2* expression (Hummel *et al*., 2004). Additionally, the decrease of ADC activity in *adc2* mutants but not in *adc1* mutants, suggests that ADC2 is making a larger contribution to arginine decarboxylation during polyamine biosynthesis (Rossi *et al*., 2015). When it comes to stress responses, *ADC1* expression is induced primarily by cold treatment, whereas *ADC2* is induced by a variety of stresses, including drought, salinity, wounding, and jasmonate treatment (Perez-Amador *et al*., 2002; Hummel *et al*., 2004; Alcazar *et al*., 2010). Moreover, whereas *ADC2* is highly upregulated, resulting in putrescine accumulation, upon inoculation of the semi-biotrophic pathogen *Pseudomonas syringae* strain DC3000, the *ADC1* transcript level responded only slightly to the infection (Kim *et al.*, 2013). However, only *ADC1* expression, also resulting in putrescine accumulation, is induced by infection with the pathogenic bacterium *Pseudomonas viridiflava* (Rossi *et al*., 2015). Taken together, these studies indicate that the two Arabidopsis ADC paralogs are specialized in their regulation and specific participation in plant stress responses.

Reduced putrescine accumulation, as well as reduced freezing tolerance, have been observed in both *adc1* and *adc2* Arabidopsis mutants (Cuevas *et al*., 2008). This phenotype can be rescued by exogenous addition of putrescine, suggesting that the importance of ADC activity results from its function in polyamine biosynthesis. Similar rescue with exogenous supply of putrescine has also been observed in the case of *P. syringae* resistance in *adc2* mutant plants (Kim *et al.*, 2013). A certain degree of functional redundancy has been shown between ADC1 and ADC2. For example, *ADC1* and *ADC2* expression is increased in *adc2* and *adc1* mutant plants, respectively, after *P. viridiflava* infection (Rossi *et al*., 2015). However, no similar reciprocal increase was observed for basal *ADC1* and *ADC2* expression under non-stressed conditions (Rossi *et al*., 2015; Sánchez-Rangel *et al*., 2016). The most unequivocal evidence of functional redundancy is the observation that Arabidopsis *adc1 adc2* double mutants are embryo

lethal, indicating that having at least one functional *ADC* gene is essential for seed development (Urano *et al*., 2005).

Several polyamine acyltransferases have been identified in plants. It has been suggested that the products have functions in a variety of plant physiological processes, ranging from providing nitrogen sinks to regulating plant development and stress responses (Bouchereau *et al*., 1999; Luo *et al*., 2009; Bassard *et al*., 2010). In one example, recent studies identified Arabidopsis *NATA1* (*N-ACETYLTRANSFERASE ACTIVITY1*; At2g39030) as an acetyltransferase that produces N^{δ}-acetylornithine and *N*-acetylputrescine from ornithine (EC 2.3.1.-) and putrescine (EC 2.3.1.57), respectively (Fig. 4.1; Adio *et al*., 2011; Lou *et al*., 2016). Putrescine acetylation by NATA1 has been shown to play a role in fine-tuning polyamine titer, thereby regulating the accumulation of H_2O_2 formed via polyamine oxidation (Lou *et al*., 2016). An adjacent gene, *NATA2* (At2g39020), is homologous to *NATA1,* encoding an enzyme with approximately 80% amino acid sequence identity. Interestingly, the *NATA1-NATA2* duplication event appears to have taken place in the lineage leading to Arabidopsis, *Arabidopsis lyrata*, and *Capsella bursapastoris*, with less closely related species, including *Brassica* spp., having only *NATA2* homologs (Lou *et al*., 2016).

Here we provide evidence that Arabidopsis ADC1 also has N^{δ}-acetylornithine decarboxylase activity, which demonstrates an alternative pathway for plant polyamine biosynthesis (Fig. 4.1). Exogenous supplementation of N^{δ}-acetylornithine leads to accumulation of *N*-acetylputrescine in an ADC1-dependent manner, which is in agreement with the *in vitro* N^{δ}-acetylornithine decarboxylase activity of ADC1. Stable isotope labelling experiments further confirm the existence of an alternate, N^{δ}-acetylornithine-dependent polyamine biosynthesis pathway in Arabidopsis. The relatively recent gene duplications producing *ADC1* and *NATA1*, in combination with the absence of *ODC*, in Arabidopsis suggest that there is an advantage to having two distinct pathways for polyamine biosynthesis in plants.

Results

Arabidopsis converts N^{δ}-acetylornithine into *N*-acetylputrescine

Both N^{δ}-acetylornithine and *N*-acetylputrescine become very abundant in Arabidopsis leaves after elicitation with methyl jasmonate (MeJA) or coronatine, a *P. syringae* toxin that is a

molecular mimic of conjugated jasmonate-isoleucine (Adio *et al*., 2011; Lou *et al*., 2016). However, Lou *et al*. (2016) showed that NATA1 acetylates ornithine more efficiently than putrescine *in vitro*. Hence, we hypothesized that, in addition to NATA1-dependent acetylation of putrescine, there is an additional pathway for *N*-acetylputrescine formation. Given that N^{δ}-acetylornithine and *N*-acetylputrescine have similar backbone structures, and MeJA-induced accumulation of N^{δ}-acetylornithine disappears over the period of a few days (Adio *et al*., 2011), we hypothesized that N^{δ}-acetylornithine can be decarboxylated to form *N*-acetylputrescine.

In initial experiments, we exogenously supplied N^{δ}-acetylornithine to detached Arabidopsis rosette leaves via their petioles. After three days of MeJA-treatment, accumulation of *N*-acetylputrescine in *nata1* mutant leaves fed with N^{δ}-acetylornithine reached a level comparable to that of wildtype Columbia-0 (Col-0) leaves (Fig. 4.2A). Consistent with previously published results, no *N*-acetylputrescine was detected in *nata1* mutant leaves when water was supplied as a control (Fig. 4.2; Lou *et al*., 2016). This observation suggested the existence of an as yet unexplored metabolic pathway for converting N^{δ}-acetylornithine to *N*-acetylputrescine.

ADC1 converts N^{δ}-acetylornithine to *N*-acetylputrescine

We further hypothesized that one of the two Arabidopsis arginine decarboxylases, ADC1 or ADC2, may also decarboxylate N^{δ}-acetylornithine. In fact, it has been suggested that the two Arabidopsis *ADC* genes, *ADC1* and *ADC2*, are the result of duplication and regulatory neofunctionalization (Hummel *et al.*, 2004). However, to the best of our knowledge, shifts in ADC substrate specificity have not been documented.

Arabidopsis *nata1 adc1* and *nata1 adc2* double mutants were generated to test whether a functional ADC is critical for generating *N*-acetylputrescine from N^{δ}-acetylornithine. Consistent with previous experiments involving *nata1* mutants (Adio *et al*., 2011; Lou *et al*., 2016), neither *nata1 adc1* nor *nata1 adc2* double mutants accumulated N^{δ}-acetylornithine and *N*-acetylputrescine above background levels in response to MeJA treatment. However, with exogenous addition of N^{δ}-acetylornithine, *N*-acetylputrescine accumulation increased in *nata1 adc2* leaves but not in *nata1 adc1* leaves (Fig. 4.2B). This indicates that ADC1 plays a critical role in converting N^{δ}-acetylornithine to *N*-acetylputrescine. To further confirm this hypothesis, *in vitro* assays were performed with Arabidopsis ADC1 and ADC2, which were purified after

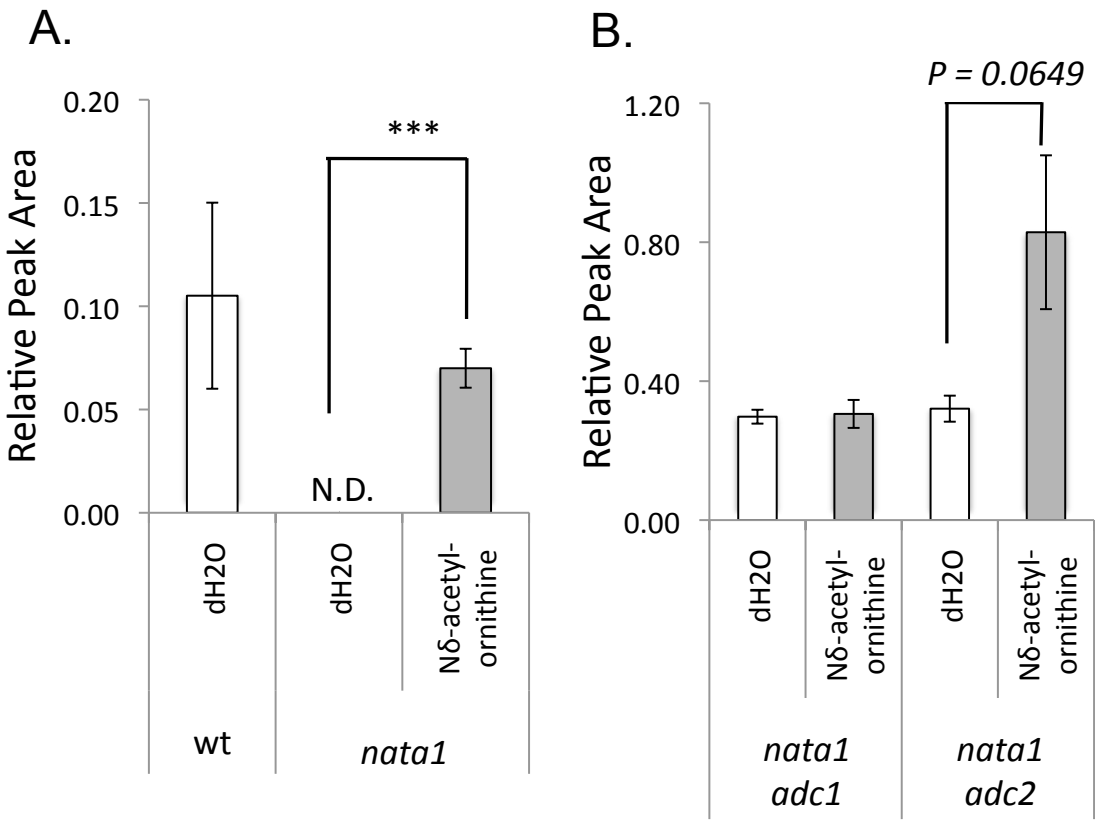

Figure 4.2. (A) Accumulation of *N*-acetylputrescine in wildtype Arabidopsis and *nata1* mutants, with and without exogenous addition of N^{δ}-acetylornithine. Mean +/- s.e. of N = 3-5. (B) Exogenous addition of N^{δ}-acetylornithine increases putrescine accumulations in *nata1 adc2*, but not *nata1 adc1* plants. Mean +/- s.e. of N = 4. ***P < 0.005, two tailed *t*-test.

transient gene expression in *Nicotiana benthamiana*. Consistent with the *in planta* results, ADC1 converted N^{δ}-acetylornithine into *N*-acetylputrescine. However, no enzymatic activity was observed with ADC2 relative to the empty vector control construct (Fig. 4.3). Interestingly, we also noticed accumulation of putrescine during ADC1 *in vitro* enzyme assays, following the accumulation of high amounts of *N*-acetylputrescine (data not shown). Taken together, these results indicate that ADC1, but not ADC2, is an N^{δ}-acetylornithine decarboxylase that can utilize N^{δ}-acetylornithine as a substrate to produce *N*-acetylputrescine. Arginine decarboxylase activity was tested for both purified enzymes to confirm their functionality.

The *ADC* duplication co-occurs with a *NATA* gene duplication in *ODC*-deficient Brassicaceae

The presence of two *ADC* genes has been suggested to be the norm in the Brassicaceae and coincides with the loss of *ODC* in this plant family (Galloway *et al*., 1998). However, a maximum likelihood-based phylogenetic analysis of predicted protein sequences sources shows that the Arabidopsis *ADC* gene duplication resulted in a more diverged ADC1 protein than the duplications found in several other Brassicaceae (Fig. 4.4A). Although several other Brassicaceae have two or more *ADC2* homologs, Arabidopsis *ADC1* homologs were only found in the genomes of *A. lyrata*, *Capsella rubella*, and *Camelina sativa* (Fig. 4.4A). Interestingly, this gene duplication pattern is similar to the *NATA1* and *NATA2* tandem duplication, which also is only found in Arabidopsis and close relatives (Fig. 4.4B; Adio *et al*., 2011; Lou *et al*., 2016). Moreover, using the ODC amino acid sequence from *Carica papaya* as a probe, we were unsuccessful in finding any BLAST hits in the Brassicaceae after screening publicly available genome and protein databases. Therefore, we hypothesized that the concurrent duplication of NATA and ADC compensates for the loss of ODC by providing a different pathway for polyamine biosynthesis in Arabidopsis*, A. lyrata*, *C. rubella* and *C. sativa*.

NATA1 and ADC1 provide an alternative pathway for polyamine biosynthesis

The predicted alternative pathway for polyamine biosynthesis starts with NATA1 synthesizing N^{δ}-acetylornithine from ornithine, providing substrate for ADC1-mediated decarboxylation, generating *N*-acetylputrescine. *N*-acetylputrescine would then be further converted to putrescine by an as yet uncharacterized Arabidopsis de-acetylating enzyme (Fig. 4.1). To test this hypothesis, we synthesized [$^{13}C_7$]N^{δ}-acetylornithine for stable isotope labeling

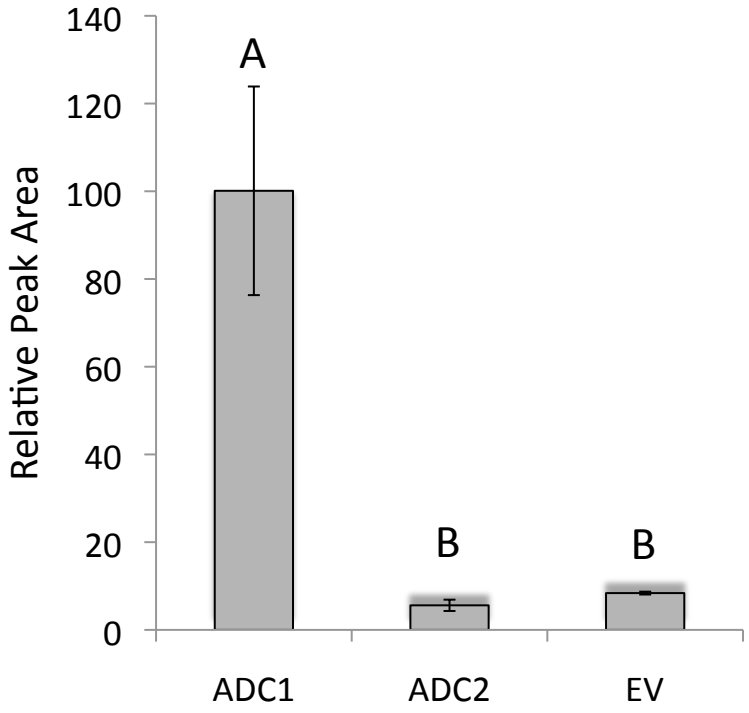

Figure 4.3. ADC1, but not ADC2, converts N^{δ}-acetylornithine to *N*-acetylputrescine. Enzyme activity was measured by LC-MS. Mean +/- s.e. of N = 3. EV = empty vector. Different letters indicate significant differences, $P < 0.05$, ANOVA followed by Tukey's HSD test.

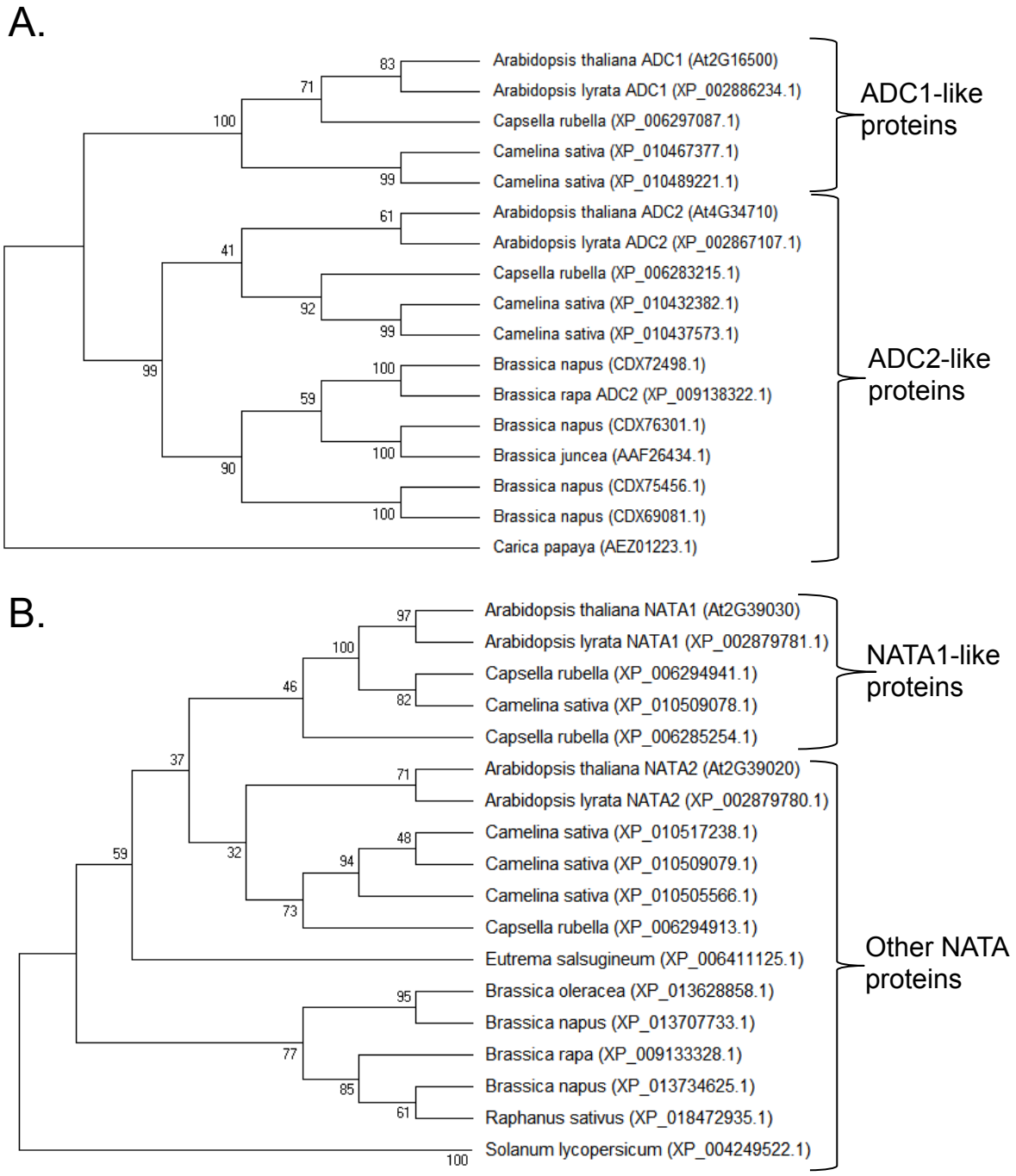

Figure 4.4 (A) Dendrogram of ADC proteins in cruciferous plants. *Carica papaya* was chosen as an outgroup. (B) Dendrogram of NATA proteins in cruciferous plants. As no predicted NATA protein sequences were found for *C. papaya*, *Solanum esculentum* was used as an outgroup.

experiments. We hypothesized that addition of [$^{13}C_7$]N^{δ}-acetylornithine to Arabidopsis leaves would result in the synthesis of [$^{13}C_6$]*N*-acetylputrescine and/or [$^{13}C_4$]putrescine (Fig. 4.5A).

Uptake of [$^{13}C_7$]N^{δ}-acetylornithine via the petioles of detached leaves resulted in the accumulation of both [$^{13}C_6$]*N*-acetylputrescine and [$^{13}C_4$]*N*-acetylputrescine (Fig. 4.5B). The ratio of [$^{13}C_6$]*N*-acetylputrescine to [$^{13}C_4$]*N*-acetylputrescine (0.12) is twice the ratio that would be expected based on natural isotope abundance (less than 0.067), suggesting that accumulation of [$^{13}C_6$]*N*-acetylputrescine is the direct result of [$^{13}C_7$]N^{δ}-acetylornithine decarboxylation. Although it is possible that [$^{13}C_6$]*N*-acetylputrescine is formed by re-acetylation of [$^{13}C_4$]putrescine with [$^{13}C_2$]acetyl-CoA derived from [$^{13}C_7$]N^{δ}-acetylornithine, this seems unlikely because the [$^{13}C_2$]acetyl group would get diluted out by unlabeled acetyl-CoA in the course endogenous plant metabolism.

The observed accumulation of [$^{13}C_4$]putrescine in wildtype Arabidopsis treated with [$^{13}C_7$]N^{δ}-acetylornithine (Fig. 4.5C) could result from either de-acetylation of [$^{13}C_6$]*N*-acetylputrescine or synthesis via the agmatine-dependent pathway (Fig. 4.1). Consistent with the latter hypothesis, we observed an increase in [$^{13}C_5$]ornithine in our labeling experiment (Fig. 4.5D), which could serve as a precursor for [$^{13}C_4$]putrescine biosynthesis via the agmatine-dependent pathway (Fig. 4.1).

To determine which of the two known pathways for *N*-acetylputrescine formation is primarily responsible for the rapid increase of *N*-acetylputrescine after MeJA treatment (Lou *et al*., 2016), we repeated the ^{13}C-labeling experiment with MeJA treatment. After 48 hours of [$^{13}C_7$]N^{δ}-acetylornithine uptake and MeJA elicitation, [$^{13}C_4$]*N*-acetylputrescine became abundant (Fig. 4.5E). However, the amount of [$^{13}C_6$]*N*-acetylputrescine remained similar to that in samples without MeJA treatment. Taken together, our observations indicate that *N*-acetylputrescine is rapidly formed from putrescine via NATA1 activity upon MeJA treatment, as suggested by Lou *et al*. (2016). However, in the absence of MeJA treatment, *N*-acetylputrescine can also be formed through decarboxylation of N^{δ}-acetylornithine by ADC1.

ADC1 and NATA1 are localized to the Endoplasmic Reticulum

It has been suggested that, with the majority of amino acid sequence differences being on the *N*-terminal end of ADC1 and ADC2 (Hanfrey *et al.*, 2001; Fig. 4.6), these two enzymes might differ in their subcellular location. In particular, unlike ADC2, ADC1 does not have a

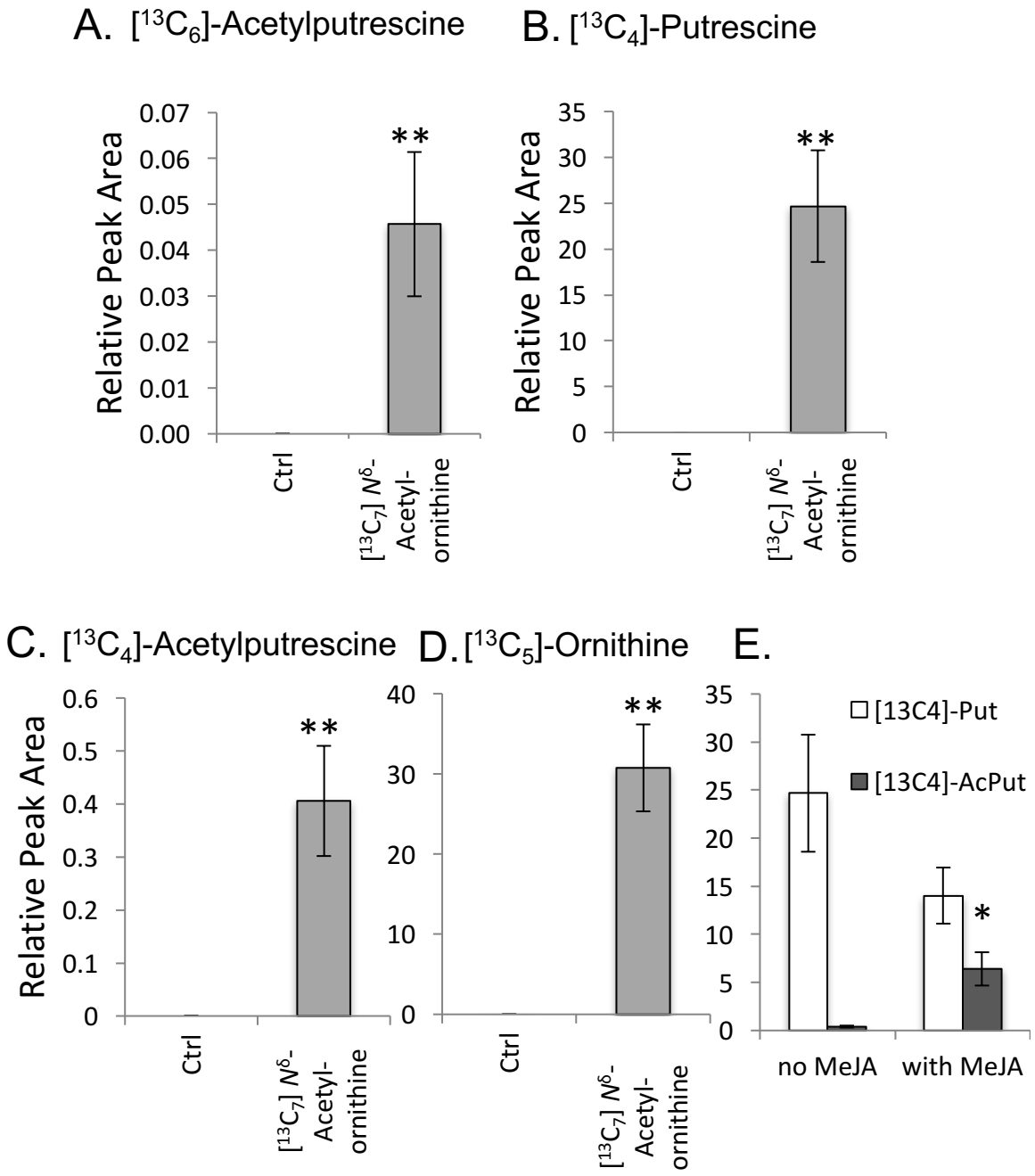

Figure 4.5. Accumulation of isotope-labeled products after uptake of [$^{13}C_7$]N^{δ}-acetylornithine by detached Arabidopsis leaves via the petioles. (A) [$^{13}C_6$]-acetylputrescine, (B) [$^{13}C_4$]-putrescine, (C) [$^{13}C_4$]-acetylputrescine, and (D) [$^{13}C_5$]-ornithine. (E) [$^{13}C_4$]-putrescine and [$^{13}C_4$]-acetylputrescine accumulation before and after MeJA treatment. Mean +/- s.e. of N = 6. *P < 0.05, **P < 0.01, two tailed *t*-test comparing to control.

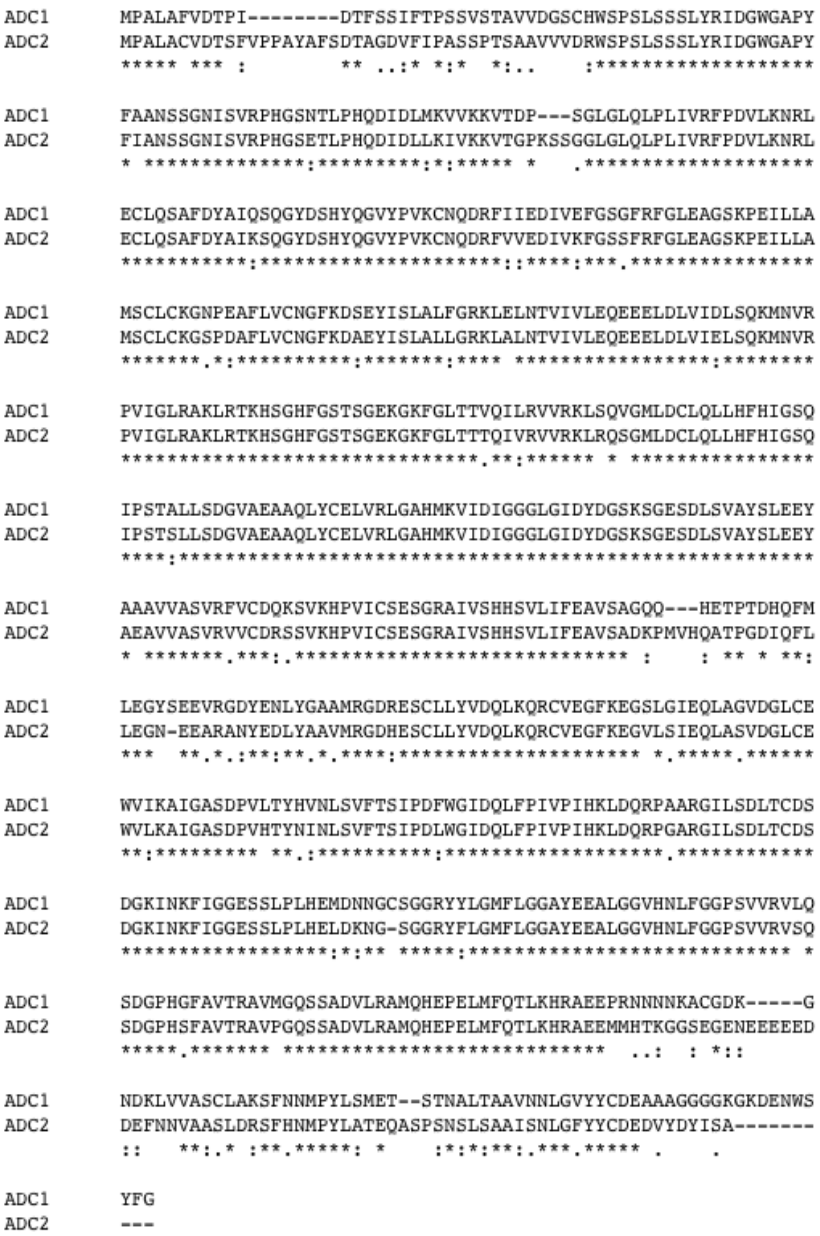

Figure 4.6. Amino acid sequence alignment of Arabidopsis ADC1 and ADC2

predicted transit peptide for localization to the chloroplast (Borrell *et al.*, 1995; Hanfrey *et al.*, 2001). To identify the intracellular location of ADC1 and NATA1, we generated GFP-fused constructs and performed analysis by confocal microscopy. Chlorophyll fluorescence was used as a marker to localize the chloroplasts within mesophyll cells (Fig. 4.7A, D). Transformed cells showed that ADC1 and NATA1 were both localized to discrete regions within the cytoplasm (Fig. 4.7B, F). Co-transformation of GFP-ADC1 and GFP-NATA1 with an mCherry endoplasmic reticulum marker (Fig. 4.7C, G) indicated that both ADC1 and NATA1 are localized to the endoplasmic reticulum (ER) (Fig. 4.7D, H). These experiments suggest that ADC1 has direct access to N^{δ}-acetylornithine produced by NATA1.

The alternative pathway is contributing to pathogen-induced polyamine biosynthesis

To determine whether the NATA1 and ADC1-mediated alternative pathway for polyamine biosynthesis is physiologically relevant, we applied *Pseudomonas syringae* infection, a biotic stress that induces both *NATA1* and *ADC1* expression. Two days after *P. syringae* infiltration, *AIH* and *CPA* gene expression in *nata1*, *nata1 adc1*, and *nata1 adc2* mutant plants was induced to a higher level than in wildtype Arabidopsis (Fig. 4.8A,B). This transcriptional induction of gene expression in the arginine-dependent pathway of polyamine biosynthesis might be compensating for the lack of polyamine biosynthesis from N^{δ}-acetylornithine.

Discussion

Our finding that Arabidopsis ADC1 catalyzes N^{δ}-acetylornithine decarboxylation to *N*-acetylputrescine identifies a new pathway for polyamine biosynthesis in Arabidopsis. We showed that ADC1, but not ADC2, has this expansion of substrate specificity. Interestingly, gene duplication and gain of function to form both *ADC1* and *NATA1* is observed in only a subset of the Brassicaceae, including Arabidopsis, *A. lyrata, C. rubella,* and *C. sativa* (Fig. 4.4). We also show that, likely due to the loss of its transit peptide, Arabidopsis ADC1 is localized in the ER instead of in the chloroplast, as is the case for oat ADC, tobacco ADC (which can also be located in the nucleus), and Arabidopsis ADC2 (Borrell *et al.*, 1995; Hanfrey *et al.*, 2001; Bortolotti *et al.*, 2004; Ariyaratne, 2014). This allows ADC1 and the product of NATA1 activity, N^{δ}-acetylornithine, to come into direct contact, thereby enabling the N^{δ}-acetylornithine-dependent polyamine pathway to develop in Arabidopsis and closely related Brassicaceae.

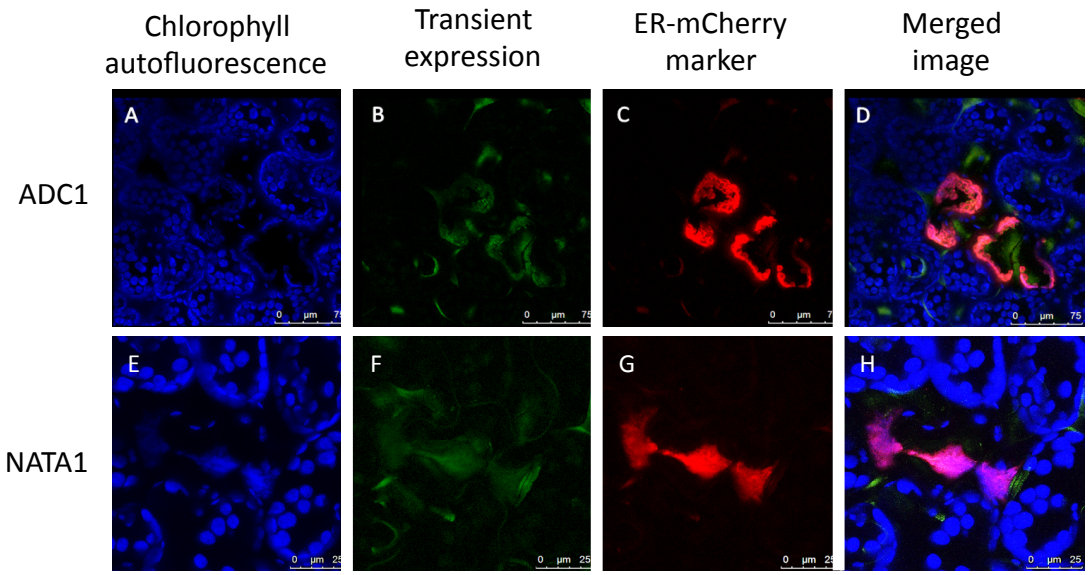

Figure 4.7. ADC1 and NATA1 are localized in the endoplasmic reticulum. ADC1-GFP, NATA1-GFP, and ER-localized mCherry were expressed in *N. benthamiana* leaves. (A, E) chlorophyll autofluorescence, have been false-colored blue. (B, F) ADC1-GFP, (C, G) ER-mCherry, and (D, H) merged image. In cell layers where both ADC1-GFP and ER-mCherry are expressed, the image is pink.

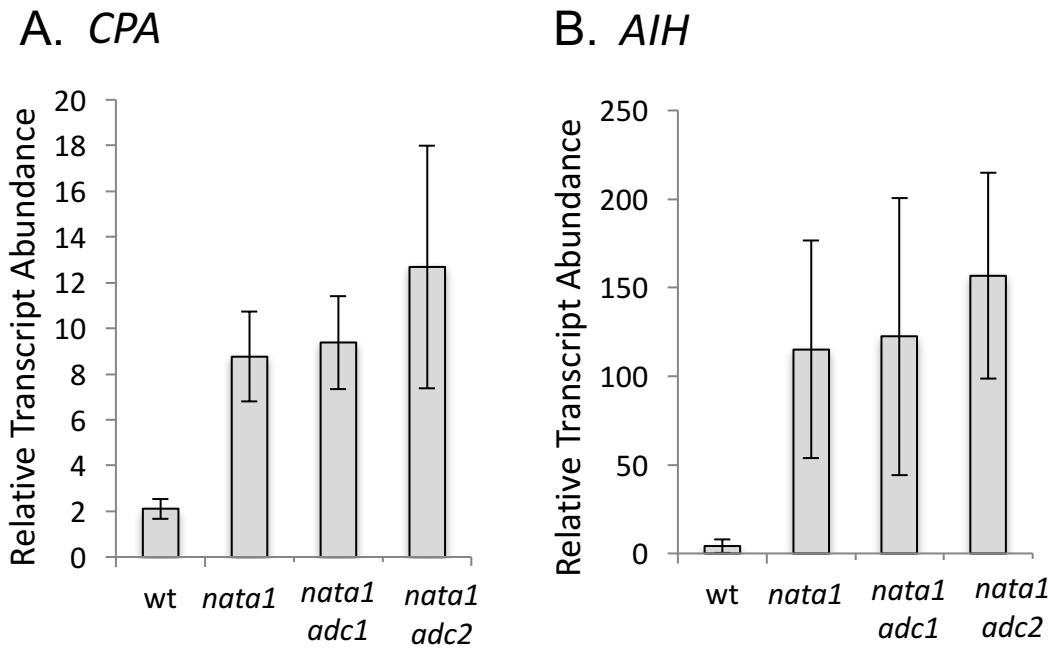

Figure 4.8. Relative to wildtype plants, (A) *AIH* and (B) *CPA* expression is increased in *nata1, nata1 adc1*, and *nata1 adc2* knockout mutants two days after *Pseudomonas syringae* infiltration. Mean +/- s.e. of N = 4-6.

Although the arginine-dependent pathway for putrescine biosynthesis has been reported in plants, bacteria, and invertebrates (Li *et al.*, 1994; Reis and Regunathan, 2000; Kusano *et al.*, 2008), it has long been regarded as the alternative pathway for ornithine-dependent pathway that is more ubiquitously found in plants (Kusano *et al.*, 2008). In fact, the arginine-dependent polyamine biosynthesis pathway in plants may be the result of endosymbiotic gene transfer from a cyanobacterium, the progenitor of chloroplast, to host nuclear genome (Illingworth *et al.*, 2003; Kusano *et al.*, 2008). Plants benefit from having both the arginine- and ornithine-dependent polyamine biosynthesis pathways, but can survive with either one of them alone. For example, none or only remnant genes of the arginine pathway enzymes for polyamine biosynthesis have been found in the sequenced genomes of the green algae *Chlamydomonas*, *Volvox*, and *Chlorella* (Fuell *et al.*, 2010). On the other hand, no ODC orthologs have been discovered in the moss *Physcomitrella patens* or in the Brassicaceae (Galloway *et al.*, 1998; Fuell *et al.*, 2010). Considering that polyamines are essential for plant survival (Urano *et al.*, 2005; Kusano *et al.*, 2008), it is not too surprising that both ODC-less *P. patens* and Brassicaceae plants have two or more ADC paralogs, while the ADC-less green alga *Chlamydomonas reinhardtii* has two ODC paralogs (Galloway *et al.*, 1998; Fuell *et al.*, 2010).

Using Brassicaceae cDNA sequences, Galloway *et al*. (1998) grouped the duplicated copies of *ADC*s into two clades, *ADC1* and *ADC2*, with Arabidopsis *ADC1* and *ADC2* belonging to the corresponding clades, respectively. Using predicted amino acid sequences from publicly available Brassicaceae genomes, we obtained similar results suggesting separation between Arabidopsis *ADC1* and *ADC2.* However, our results also show that Arabidopsis ADC1 is more diverged from the other duplicated ADC copies in the Brassicaceae. For instance, all four ADC copies found in *Brassica napus* are homologous to Arabidopsis ADC2 and none to ADC1. In fact, Arabidopsis *ADC1* homologues are only found in close relatives of Arabidopsis, including *A. lyrata*, *C. rubella*, and *C. sativa*. Interestingly, the list of Brassicaceae having *ADC1* homologues coincides with those that have the *NATA1-NATA2* tandem gene duplication. More genome sequencing will be required to determine the universality of these correlated *ADC1-ADC2* and *NATA1-NATA2* gene duplications in the Brassicaceae.

The *ADC1-ADC2* duplication has been suggested to compensate for the loss of *ODC* (Galloway *et al.*, 1998; Hanfrey *et al.*, 2001). In Arabidopsis, whereas *ADC2* is strongly stress-inducible, *ADC1* expression is more similar to the constitutive *ODC* expression observed in other

plant species (Hanfrey *et al.*, 2001; Hummel *et al.*, 2004; Cuevas *et al.*, 2008; Alcazar *et al.*, 2010). Moreover, much of the amino acid sequence differences between Arabidopsis ADC1 and ADC2 are at the *N*-terminus (Hanfrey *et al.*, 2001), suggesting variation in subcellular location, and hence, differences in the accessible metabolite pool. We show that ADC1 is localized on the endoplasmic reticulum, giving it access to cytosolic substrates. This is similar to mammalian ODC, which also is located in the cytoplasm (Cohen, 1998). Since ADC1 functions as both an N^{δ}-acetylornithine and an arginine decarboxylase, we do not rule out that this location also provides a cytosolic arginine-dependent biosynthesis pathway for polyamine biosynthesis as an alternative to the ADC2-catalyzed one in the chloroplasts

Our results show that, in Brassicaceae that have both *ADC1* and *NATA1*, as a result of the respective duplication events, the encoded enzymes can form an alternative pathway for polyamine biosynthesis. NATA1 acetylates ornithine to provide a substrate for ADC1, which catalyzes N^{δ}-acetylornithine decarboxylation to *N*-acetylputrescine. However, it is not yet clear how N^{δ}-acetylornithine and *N*-acetylputrescine are converted to ornithine and putrescine, respectively. However, the fact that we measured putrescine after large amounts of *N*-acetylputrescine accumulation during *in vitro* assays suggests that a non-enzymatic degradation reaction might be playing a role. It is also possible that NATA1 functions in a bi-directional manner in Arabidopsis, converting *N*-acetylputrescine to putrescine. On the other hand, one or more of the many deacetylases and transaminases in Arabidopsis might catalyze the reaction.

The pathway for *N*-acetylputrescine formation indicates an advantage to having more than one polyamine biosynthesis mechanism. For example, although not being able to convert *N*-carbamoylputrescine to putrescine, the *cpa* knockout Arabidopsis line is viable and shows no obvious growth defect comparing to wild type. The essential polyamines in *cpa* mutants are likely made via N^{δ}-acetylornithine pathway and/or the ADC-agmatine aminohydrolase pathway (Ariyaratne, 2014). Conversely, *nata1*, *nata1 adc1* and *nata1 adc2* knockout mutants showed higher *CPA* and *AIH* transcript abundance than wildtype Arabidopsis, indicating a shift in the polyamine pathways when this route to polyamine synthesis is blocked.

The N^{δ}-acetylornithine and arginine pathways might also contribute to polyamine biosynthesis differently under different circumstances, as indicated by the different regulation of genes in the pathway. In fact, together with the MeJA-inducibility of *NATA1*, the N^{δ}-

acetylornithine pathway might contribute to polyamine-mediated stress responses and signaling. N^{δ}-Acetylornithine and *N*-acetylputrescine both can function as polyamine sinks and, at the same time, allow a slower biosynthesis of putrescine from its precursors. In mammals, polyamine acetylation is a well-known stress response, whereby cells control the polyamine homeostasis through transport of the deprotonized polyamines across membranes, resulting in regulatory effects on many aspects of animal metabolism (Casero and Pegg, 1993; Tavladoraki *et al.*, 2012). A similar scenario could occur in plant cells, where the production of N^{δ}-acetylornithine and *N*-acetylputrescine has been related to pathogen resistance and the defense-related hydrogen peroxide burst (Lou *et al.*, 2016).

Our study suggests that the duplication and neofunctionalization of *ADC1-ADC2* and *NATA1-NATA2* in Arabidopsis provide a previously unknown pathway for polyamine biosynthesis. As both enzymes in this pathway are stress-induced, it would be interesting to elucidate how the pathway interacts with plant defenses. Polyamine biosynthesis via N^{δ}-acetylornithine may be limited to Arabidopsis and close relatives, as indicated by the restricted gene duplications within Brassicaceae. Nevertheless, our research highlights the importance of conjugated polyamines, in particular acetylated polyamines, and may lead to the discovery of new functions for these metabolites in Arabidopsis and other plant species. Including acetylated polyamines, in addition to the more-commonly-studied free ones, in future studies of plant polyamine metabolism will shed new light on the many interconnected phenotypes associated with this group of metabolites.

Methods

Plant material and growth conditions

Seeds of wildtype Columbia-0 Arabidopsis, *nata1* (GK-256F07), *adc1* (CS9658), and *adc2* (CS9660) were obtained from the Arabidopsis Biological Resource Center (www.arabidopsis.org). Plants were grown in Cornell mix (by weight 56% peat moss, 35% vermiculite, 4% lime, 4% Osmocoat slow-release fertilizer [Scotts, Marysville, OH], and 1% Unimix [Scotts]) in 20x40-cm nursery flats in Conviron growth chambers with a photosynthetic photon flux density of 200 mmol m^{-2} s^{-1} and a 16:8 h day:night photoperiod, at 23°C with a 50% relative humidity.

Creation of double mutant lines

The *nata1 adc1*, *nata1 adc2* double mutants were made by crossing a *nata1* mutant (GK-256F07) and *adc1* (CS9658) and *adc2* (CS9660) mutants, respectively. Arabidopsis DNA was extracted using the CTAB (cetyltrimethylammonium bromide) method, and the double mutants were confirmed in their respective F2 populations by PCR (Primers in Supplemental Table 1).

Mass spectrometry

Polyamines were extracted and derivatized with a 4-(N,N-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F) method modified from Tsutsui *et al.*, (2013) was used prior to HPLC-MS detection. In short, plant tissue was pulverized in liquid nitrogen and extracted with 25 mM HCl (5 ml mg^{-1} of tissue) containing 25 µM 1,6-hexanediamine as an internal standard. After centrifuging at 15,000 x g at 10°C for 20 min, the supernatants (12 µL each) were mixed with 24 µL of 0.2 M sodium tetraborate. Equal volumes of DBD-F solution, dissolved in acetonitrile, were added to each sample prior to 30 min incubation at 60°C. The reaction mixtures were filtered and analyzed on a Waters® ACQUITY UPLC® BEH C18 column (Waters) using a Thermo Q-Exactive Orbitrap coupled to Dionex ultra-high-pressure chromatography systems for reverse-phase separation with column temperature set at 40 °C. The start condition were 80% solvent A (0.1% formic acid in water) and 20% solvent B (acetonitrile) with flow rate at 0.5 mL/min. The elution gradient was as follows: ramp to 90% solvent B at 5.5 min with curve set to 5 and hold at 90% solvent B till 7 min. The detection conditions were as follows: Heated electrospray ionization mode; 4000 V spray voltage; 320 °C capillary temperature; 70 units sheath gas flow rate; mass range of m/z 80 - 1200 Da. Analytical software (Therm Xcalibur v3.0) was used for the system control and data processing.

^{13}C labeling experiments

[$^{13}C_7$]N^{δ}-Acetylornithine was synthesized and purified as describe previously (Adio *et al*., 2011), but using [$^{13}C_5$]ornithine and phenyl P-nitrophenyl $^{13}C_2$-acetate/$^{13}C_2$-acetate. The purity of the synthesized compound was verified by HPLC and GC-MS.

Protein extraction and *in vitro* assays

For protein purification, ADC1 and ADC2 were cloned behind the cauliflower mosaic virus 35S promoter in the T-DNA binary vector pYL436 as described by Rubio *et al.*, 2005) and transformed into *Agrobacterium tumefaciens* strain GV3101 for *N. benthamiana* transient expression. Primers used for cloning are listed in Supplementary Table S4.1. A pYL436 empty vector was included as control. Proteins were further purified by immunoprecipitation as described in Brauer *et al.*, 2014, with slight modification. In short, fully expanded *N. benthamiana* leaves from young and flower-less plants were infiltrated with a mixture of *A. tumefaciens* strains carrying the desired constructs and turnip crinkle virus capsid protein (P38) using a 1 ml needle-less syringe. Leaf tissue was collected three days after infiltration, flash frozen, and ground to fine powder in liquid nitrogen. All of the following purification steps were carried out in a 4°C cold room. After mixing with extraction buffer (100 mM Tris-HCL, pH 7.5, 100 mM NaCl, 5 mM ethylene-diamine-tetra-acetic acid (EDTA), 5 mM ethylene glycol tetraacetic acid (EGTA), 0.1% Triton X-100, 1% β-mercaptoethanol, 10% glycerol and 1mM PMSF), samples were centrifuged at 3,600 x g for 10 min. The supernatant was mixed with IgG Sepharose® 6 Fast Flow (GE Healthcare Lifescience; http://www.gelifesciences.com/) and incubated with 360° rotation for 2-4 hours. Beads were washed three times with the same buffer containing 500 mM NaCl and an additional time with cleavage buffer (50 mM Tris-HCL, pH 7.0, 150 mM NaCl, 1 mm EDTA, 1 mM dithiothreitol, 1% Triton X-100), before being incubated with Turbo3C™ Protease (Accelagen; http://www.accelagen.com/) in cleavage buffer overnight. Supernatants were collected the next morning and incubated with Glutathione-Superflow Resin (Clontech; http://www.clontech.com/) for 1 hour with rotation. Proteins purified using this method were aliquoted and stored as a 25% glycerol sample at -80 °C. Prior to *in vitro* experiments, the total protein content was quantified by Bradford assay and a single protein band could be observed by SDS-polyacrylamide gel electrophoresis and silver staining.

Enzymatic activity toward potential substrates was determined using 2 μg purified protein under the following conditions: 100 mM Tris-HCl (pH 7.5), 2 mM EDTA and 10% glycerol at 30°C, with substrate concentration of 5 mM. Commercially available polyamines and acetylated polyamines, including ornithine, putrescine, spermidine, spermine, *N*-acetylputrescine, N^1-acetylspermidine, N^1-acetylspermine and 1,3-diaminepropane, were used as

substrates. Samples were further derivitazed and analyzed by LC-MS for measurement of the potential products. Arginine was included as a control for enzyme activity of ADC1 and ADC2.

Phylogenetic trees

Arabidopsis ADC and NATA homologs in the Brassicaceae were obtained by comparison to genomic data in GenBank and Phytozome v.11.0 (https://phytozome.jgi.doe.gov/). The ADC homolog of *Carica papaya* and the closest NATA homolog in *S. lycopersicum* were included as outgroups for the respective phylogenetic trees. After amino acid sequences were aligned using Clustal Omega (Sievers *et al.*, 2011), rooted phylogeny trees were constructed with MEGA5 using a maximum-likelihood algorithm (Jones-Taylor-Thornton probability model, gamma distributed rates among sites). A bootstrap resampling analysis with 1,000 replicates was performed to evaluate the tree topology.

***Pseudomonas syringae* infection**

Pseudomonas syringae strain DC3000 was obtained from N. Clay (Yale University, New Haven, CT), and an infection experiment performed as described by Lou *et al*. (2016). In short, bacteria were cultured at 30°C in LB broth supplemented with 50 μg ml^{-1} rifampicin and 50 μg ml^{-1} kanamycin. Overnight cultures were centrifuged at 3000 x g for 10 min, resuspended and diluted in water to approximately 10^5 colony forming units (CFU) ml^{-1} before infiltration into three-week-old Arabidopsis rosette leaves with a needle-less syringe. 8-mm diameter leaf discs were collected 2 days after infiltration and submerged in 1 ml sterile water with 0.01% Tween 20 for 4 h to allow equilibration of the bacteria between the apoplastic space of the leaf disks and the surrounding water, as described previously (Anderson *et al.*, 2006). Serial dilutions of the suspension were spotted on a Luria-Bertani (LB) agar plate supplemented with 50 μg ml^{-1} rifampicin and bacterial colonies were counted after 2 days of incubation at 30°C.

Subcellular Localization of ADC1 and NATA1

ADC1 and *NATA1* were amplified from Arabidopsis genomic DNA and cloned into a GATEWAY® destination vector, pYL436. Primers used are listed in Supplementary Table S4.1. GATEWAY® technology was used to transfer *ADC1* and *NATA1* into the GFP-fusion expression vector pGWB5 (Nakagawa *et al.*, 2007). The mCherrry-ER marker (ER-rbCD3960;

Nelson *et al.*, 2007) was obtained from Arabidopsis Biological Resource Center. The *A. tumefaciens* strain GV3101 was used to deliver the fluorescent tagged proteins into *N. benthamiana* leaves (Sparkes *et al.*, 2006). Samples were imaged using a Leica TCS SP5 laser scanning confocal microscope (Leica Microsystems, Bannockburn, IL) and the Leica Application Suite Advanced Fluorescence (LASAF) program. Images were acquired in the XYZ plane in 1 µm steps with a 63X oil objective (NA 1.40) using the sequential scan mode to eliminate any spectral overlap in the individual fluorophores. GFP was excited at 488 nm and detected at 510 nm. mCherry was excited at 561 nm and detected at 610 nm. Plastids were excited at 633 nm and detected at 670 nm. GFP signals were false-colored green, mCherry fluorescence was false-colored red, and chlorophyll autofluorescence was false-colored blue. Background fluorescence from untransformed leaves of plants at similar laser excitation settings was acquired and subtracted from the images to identify fluorescence generated by tagged proteins. ImageJ was used to merge the images (Schneider *et al.*, 2012). Images were acquired from different infiltrated leaf sections at 12-72 h after leaf inoculation.

Quantitative RT-PCR

Relative transcript abundances of AIH (At5g08170) and CPA (At2g27450) were analyzed and compared by qRT-PCR using actin (At3g18780) as an endogenous control. Gene-specific primers were designed using Primer-BLAST(www.ncbi.nlm.nih.gov/tools/primer-blast/) and are listed in Supplementary Table S4.1. Rosette leaf samples were ground in liquid nitrogen and total RNA was extracted using the SV total RNA isolation system (Promega, www.promega.com). After quantification with a Nanodrop system (www.nanodrop.com), 1 µg of total RNA was reverse transcribed using SMART® MMLV Reverse Transcriptase (Clontech, www.clontech.com) using oligo$(dT)_{15}$ primers. Following cDNA synthesis, the samples were diluted in nuclease free water and used for qRT-PCR reactions with the SYBR® Green PCR master mix (Applied Biosystems, www.appliedbiosystems.com) using an Applied Biosystems 7900HT Instrument. Each reaction was carried out with the following conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 15 s and 72°C for 15 s, and final extension at 72°C for 2 min. The C_T values were quantified and analyzed according to the standard curve method.

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Supplemental Table 4.1. Primers used in this study.

Target gene name	Primer name	Forward primer sequence	Purpose
AT2G16500	ADC1LP	TCAGCTGGTCAACAACATGAG	Confirm T-DNA insertion
AT2G16500	ADC1RP	GTCTGAAACATGAGCTCAGGC	Confirm T-DNA insertion
Primer specific for Salk Institute T-DNA insertion left border	SALK-LB1.3	ATTTTGCCGATTTCGGAAC	Confirm T-DNA insertion
AT4G34710	ADC2LP	TTTCACCGGATAAACACCTTG	Confirm T-DNA insertion
AT4G34710	ADC2RP	AAATCCACTTCTGCATGGTTG	Confirm T-DNA insertion
At2g39030	NATA1LP	ATTTTCCAAGAAGAGGGATCG	Confirm T-DNA insertion
At2g39030	NATA1RP	ACTTTAAAGGACCGATCGCTC	Confirm T-DNA insertion
Primer specific for GABI-KAT T-DNA insertion left border	GK-LB	ATATTGACCATCATACTCATTGC	Confirm T-DNA insertion
At2G16500	ADC1GwFw	GGGG ACA AGT TTG TAC AAA AAA GCA GGC TTA ACA ATG CCT GCT CTA GCT TTT GTT	Cloning
At2G16500	ADC1GwRv	GGGG ACC ACT TTG TAC AAG AAA GCT GGG TAA CCG AAA TAA GAC CAA TTC TC	Cloning
At4G34710	ADC2GwFw	GGGG ACA AGT TTG TAC AAA AAA GCA GGC TTA ACA ATG CCT GCT TTA GCT TGC GTT	Cloning
At4G34710	ADC2GwRv	GGGG ACC ACT TTG TAC AAG AAA GCT GGG TAC GCA GAG ATG TAA TCG TAG AC	Cloning
At2G27450	CPAFw	GATCAAGTCGAAAAGGCAAAGCT	RT-PCR
At2G27450	CPARv	CCATCCATAGTAAGAAGCACCTTGT	RT-PCR
At5G08170	AIHFw	TCGAGAATGCAAGAGAGATCGTT	RT-PCR
At5G08170	AIHRv	CATTTTCGGCGACGGAAGTA	RT-PCR

CHAPTER FIVE

SUMMARY AND FUTURE DIRECTIONS

The research presented in this dissertation shows that Arabidopsis *NATA1* and its closest homolog, *NATA2*, both encode polyamine acetyltransferases (Fig. 5.1). These acetyltransferases compete for metabolic flux into the polyamine biosynthesis pathway and, hence, reduce H_2O_2 formation via polyamine oxidation. Through their influence on polyamine metabolism, NATA1 and NATA2 have a regulatory function in plants under stress. More specifically, as *NATA1* is jasmonate-induced and *NATA2* is heat-induced, they contribute to the regulatory function of polyamine metabolism in linking jasmonate signaling and heat stress responses, respectively, to salicylate-signaling. In addition, I showed that formation of N^{δ}-acetylornithine by NATA1 is the first step of an ADC1-dependent alternative pathway for polyamine biosynthesis in Arabidopsis, which might be using this pathway to compensate for the loss of ODC-dependent polyamine biosynthesis that is found in most other plant species. A more detailed understanding of the biosynthesis and degradation of acetylated polyamines would not only shed light on how polyamine homeostasis is achieved in a timely manner without triggering unwanted signaling, but also open the door to improving plant responses toward multiple simultaneous stresses, the real challenge that plants face in natural environments.

More studies are needed to further elucidate details of the biosynthesis and degradation of acetylated polyamines. For example, *NATA2* homologs have been found in multiple copies in many plant species. However, whether they have the same function as the Arabidopsis homologs and what evolutionary benefit they provide, is not yet clarified. When it comes to degradation, substrates tested for Arabidopsis PAOs are limited by commercial availability, and various acetylated polyamines, such as N^1N^{12}-diacetylspermine, an SSAT product frequently found in mammalian cells, have not been investigated. At the same time, substrate preferences for the ten predicted Arabidopsis CuAOs remain to be characterized.

I was not able to obtain viable *nata1 nata2* double knockout mutants, suggesting that NATA1 and NATA2 are at least partially redundant. However, given the varying substrate preferences of these enzymes, it would be interesting to study how this

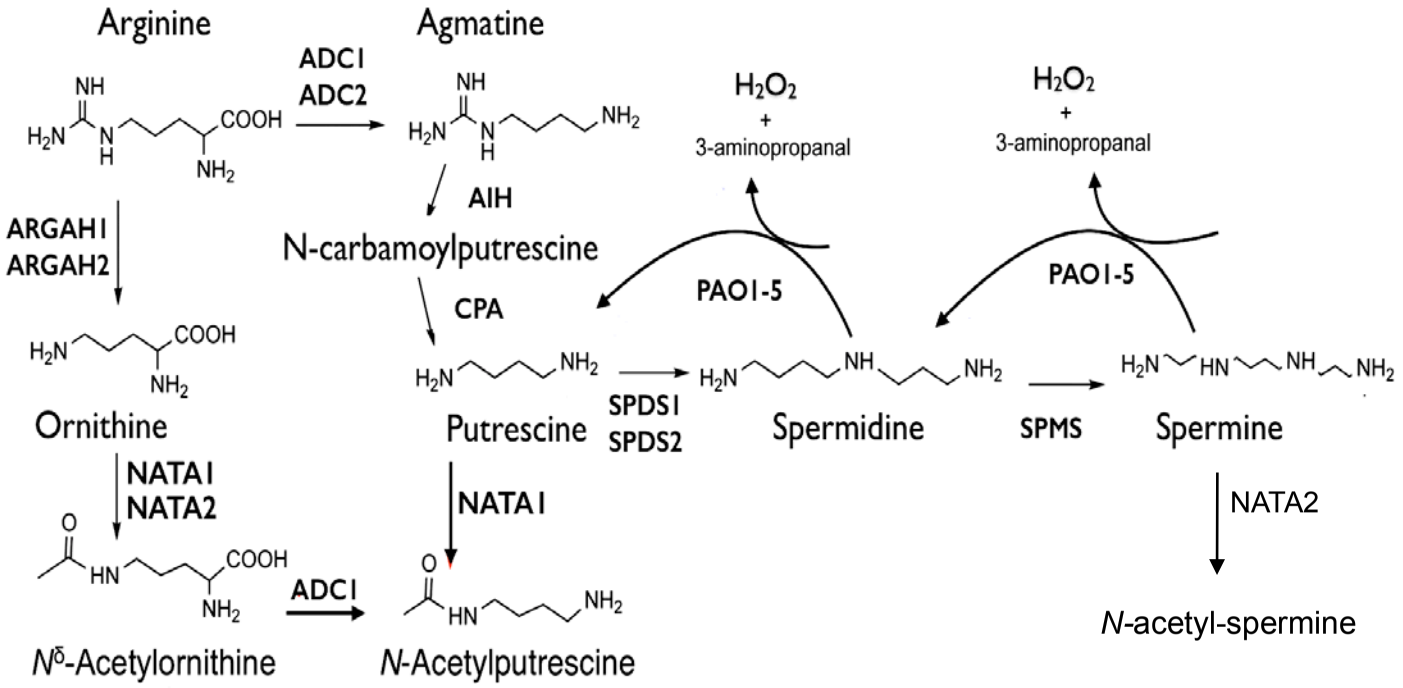

Figure 5.1. Updated pathways for polyamine synthesis and catabolism in plants. Names of known Arabidopsis proteins catalyzing the reactions are indicated.

redundancy is achieved. One hypothesis is that the products of NATA1 and NATA2 might be interconverted with one another. Investigating whether SPDS and SPMS use *N*-acetylputrescine and N^1-acetylspermidine as substrates, respectively, would provide additional fundamental information about plant metabolism. In the meantime, I was not able to rule out the possibility that acetylated polyamines, and/or their direct breakdown products, act as signaling compounds in plants. While exogenous application of acetylated polyamines is limited by commercial availability, further investigation of whether they have direct functions in plant signaling networks is warranted.

Last but not least, an expansion of the metabolites that are commonly investigated - from free polyamines to conjugated ones, as well as breakdown products - would greatly benefit the field of polyamine studies. It is now clear that polyamine titer is tightly regulated not only by its biosynthesis, catabolism, and transportation, but also by conjugation and, in particular, acetylation. Expanding the focus of relevant studies to additional polyamine-related compounds would not only provide a more accurate estimation of metabolic flux in polyamine metabolism, but also improve our understanding of how polyamine homeostasis is achieved. Accurate measurement of local polyamine concentrations, distinguishing free forms from those bound to cellular components, however, remains challenging.